Title: A Caspase Activated Protein Kinase

FIELD OF THE INVENTION

The invention relates to apoptosis and particularly a novel protein, SMAK that activates two distinct signalling pathways that are involved in mediating apoptosis. The invention includes nucleic acid molecules encoding the SMAK protein; the SMAK protein and truncations, analogs, and homologs of the protein; and uses of the protein and nucleic acid molecules in controlling cellular apoptosis. In addition, the invention includes identification of a myosin binding protein (mybp-c) that specifically binds the smak kinase domain and inactivates kinase activity.

BACKGROUND OF THE INVENTION

Programmed cell death or apoptosis is a genetically controlled process triggered by various stimuli in different cells. External stimuli such as cytokines, UV irradiation, and numerous drugs induce an apoptotic response characterized by a series of morphological changes that include cytoplasmic shrinkage, membrane blebbing, chromatin condensation, DNA fragmentation, and the formation of apoptotic bodies (Kerr et al., 1972; Raff et al., 1993; Steller, 1995). Several studies have revealed the catalytic activation of several kinases during apoptosis. For example, the cJun-aminoterminal kinase (JNK) pathway is activated in response to apoptotic triggers such as TNF-α and Fas ligand (Verheij et al., 1996; Xia et al., 1995; Yang et al., 1997). In addition, activation of other kinases, such as ASK1, RIP, or ZIP kinases, stimulates apoptosis in cultured cells (Ichijo et al., 1997; Kawai et al., 1998; Stanger et al., 1995).

The induction of apoptosis involves a proteolytic activation of a cascade of a family of cysteine proteases called caspases. Aggregation of death receptors following ligand binding activates initiator caspases, which in turn, activates downstream effector caspases through proteolytic processing (Thornberry and Lazebnik, 1998). Caspases contribute to cell death by direct inactivation of negative regulators of apoptosis and by promoting the disassembly of cellular structures such as focal adhesion complexes (Thornberry and Lazebnik, 1998). Although several caspase substrates such as nuclear lamins and poly-ADP ribose polymerase have been identified (Thornberry and Lazebnik, 1998), little is known about the biological function of the cleavage products. However, recent studies have revealed that caspase-mediated cleavage of the serine/threonine kinase PAK2 (p65PAK) generates a catalytically active fragment involved in regulating some of the morphological changes associated with apoptosis (Lee et al., 1997; Rudel and Bokoch, 1997). Caspases similarly cleave the Ste20-related kinase MST1 to release a catalytically active kinase domain that activates SAPKs as well as MKK6 and MKK7 (Graves et al., 1998; Lee et al., 1998).

Cell growth and differentiation are regulated by complex signaling processes

involving a large number of protein kinases and phosphatases. The activation or inhibition of various pathways ultimately results in the expression of specific subsets of genes directly involved in proliferation or terminal differentiation (Davis, 1993; Fanger et al., 1997). Extracellular signals acting on growth factor receptors or G-protein coupled receptors transduce their signal through a kinase cascade resulting in the activation of mitogen-activated kinases (MAPK) such as ERK1 and ERK2 . Stress-inducing agents as well as apoptotic triggers signal through the stress-activated pathway leading to the activation of JNK, p38, and other members of the stress-activated protein kinase (SAPK) family (Fanger et al., 1997). Activation of these downstream kinases requires the activation of multiple kinases and transducing molecules such as small GTPases, MAPK kinase kinases (MEKK) and MAPK kinases (MEK) (Fanger et al., 1997).

In yeast, the Ste20 serine/threonine protein kinase regulates a mitogen-activated protein kinase pathway consisting in Ste11 (MEK kinase), Ste7 (MEK) and Fus3/Kss1 (MAPK) protein kinases involved in the control of mating response (Zhao et al., 1995). Activation of Ste20 results from α factor pheromone binding to its G-protein coupled receptor and subsequent activation of Ste20 by the $\beta\gamma$ complex released from the heterotrimeric G protein. This interaction results in translocation of Ste20 to the scaffolding protein Ste5, leading to the sequential activation of Ste11, Ste7 and Fus3/Kss1 (Leberer et al., 1997). Ste20 also binds the small GTPase Cdc42, however the Cdc42 binding domain of Ste20 has been shown to be dispensable for pheromone signaling in yeast (Leberer et al., 1997).

The small GTPase proteins of the Rho subfamily mediate various cellular processes such as growth and cytoskeleton reorganization through direct binding of the activated GTP-bound forms to downstream targets (Van Aelst and D'Souza-Schorey, 1997). RhoA is required for maintenance of actin stress fibers and focal adhesions in cultured cells. These activities have been shown to be mediated by several Rho-associated protein kinases such as ROKα, p160ROCK, MRCKα, PKN, and PRK2 (Amano et al., 1997; Amano et al., 1996; Ishizaki et al., 1997; Leung et al., 1996; Leung et al., 1998; Nakagawa et al., 1996; Watanabe et al., 1996). The Cdc42 GTPase promotes the formation of actin microspikes whereas Rac1 activation induces the formation of lamellipodia or membrane ruffles. In addition to playing an important role in cellular morphology, the Rho family of GTPases regulates transcription through the JNK and p38 pathways (Fanger et al., 1997). Mammalian targets of Cdc42 and Rac1 include the PAK family of protein kinases (Van Aelst and D'Souza-Schorey, 1997). Upon binding to activated Cdc42 or Rac1, PAK is activated and translocated to focal adhesion sites (Manser et al., 1997). Expression of constitutively active αPAK causes the loss of focal adhesions and retraction of actin stress fibers to the periphery (Manser et al., 1997). In addition, PAK activation leads to stimulation of the SAPK and p38 kinase pathways (Fanger et al., 1997).

SUMMARY OF THE INVENTION

The present inventors have identified and characterized a cDNA that encodes a protein kinase that mediates apoptosis and actin stress fiber dissolution through caspase-3-cleavage and functions to activate the stress activated protein kinases (cJun-amino terminal kinase (JNK) signalling pathway).

The present invention therefore provides a purified and isolated nucleic acid molecule comprising a sequence encoding a protein having about 70% homology in the kinase domain with LOK, about 65% homology with M-NAP; and about 60% homology with ATI-46. The protein of the invention may be generally referred to as a Ste20-like kinase protein or SMAK.

In an embodiment of the invention, the purified and isolated nucleic acid molecule comprises: (i) a nucleic acid sequence encoding a SMAK protein having the amino acid sequence as shown in Figure 1; and, (ii) nucleic acid sequences complementary to (i).

In a preferred embodiment of the invention, the purified and isolated nucleic acid molecule comprises:

(i) a nucleic acid sequence encoding a SMAK protein having the nucleic acid sequence as shown in Figure 1, wherein T can also be U;

(ii) a nucleic acid sequence complementary to (i), preferably complementary to the full length nucleic acid sequence shown in Figure 1;

(iii) a nucleic acid molecule differing from any of the nucleic acids of (i) and (ii) in codon sequences due to the degeneracy of the genetic code.

The invention also contemplates: (a) a nucleic acid molecule comprising a sequence encoding a truncation of the SMAK protein, an analog or homolog of the SMAK protein or a truncation thereof; (b) a nucleic acid molecule comprising a sequence which hybridizes under high stringency conditions to the nucleic acid encoding a SMAK protein having the amino acid sequence as shown in Figure 2; and (c) a nucleic acid molecule comprising a sequence which hybridizes under high stringency conditions to the nucleic acid sequence as shown in Figure 1, wherein T can also be U, or complementary sequences thereto.

The invention further contemplates a purified and isolated double stranded nucleic acid molecule containing a nucleic acid molecule of the invention, hydrogen bonded to a complementary nucleic acid base sequence.

The nucleic acid molecules of the invention may be inserted into an appropriate expression vector, i.e., a vector which contains the necessary elements for the transcription and translation of the inserted coding sequence. Accordingly, recombinant expression vectors adapted for transformation of a host cell may be constructed which comprise a nucleic acid molecule of the invention and one or more transcription and translation elements operatively linked to the nucleic acid molecule.

The recombinant expression vector can be used to prepare transformed host

cells expressing a SMAK protein of the invention or a SMAK related protein. The expression vector can be used for gene therapy. The invention further provides one or more host cells containing a recombinant molecule of the invention. The invention also contemplates transgenic non-human mammals whose germ cells and somatic cells contain a recombinant molecule comprising a nucleic acid molecule of the invention which encodes a SMAK protein or an analog of a SMAK protein, i.e., the protein with an insertion, substitution or deletion mutation.

The invention further provides a method for preparing a SMAK protein, or a SMAK related protein utilizing the purified and isolated nucleic acid molecules of the invention. In an embodiment a method for preparing a SMAK protein is provided comprising: (a) transferring a recombinant expression vector of the invention into a host cell; (b) selecting one or more transformed host cells from untransformed host cells; (c) culturing a selected transformed host cell under conditions which allow expression of the SMAK protein; and (d) isolating the SMAK protein.

The invention further broadly contemplates a purified and isolated SMAK protein that upon caspase-3 cleavage mediates apoptosis and actin stress fiber dissolution. SMAK protein also activates the JNK pathway and has protein interaction motifs including several AU-rich motifs and an ATH domain. The SMAK protein has an approximate molecular weight of 148KDa. In an embodiment of the invention, a purified SMAK protein is provided which has the amino acid sequence as shown in Figure 2. The purified and isolated protein of the invention may be modified or activated, i.e., phosphorylated. The invention also includes truncations of the protein and analogs, homologs, and isoforms of the protein and truncations thereof.

The SMAK proteins of the invention may be conjugated with other molecules, such as proteins to prepare fusion proteins. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins.

The invention further contemplates antibodies having specificity against one or more epitopes of a SMAK protein of the invention. Antibodies may be labelled with a detectable substance and they may be used to detect the SMAK protein of the invention in tissues and cells.

The invention also permits the construction of nucleotide probes which are unique to the nucleic acid molecules of the invention and accordingly to a SMAK protein of the invention. Thus, the invention also relates to a probe comprising a sequence encoding a SMAK protein or fragment thereof. The probe may be labelled, for example, with a detectable substance and it may be used to select from a mixture of nucleotide sequences a nucleotide sequence coding for a protein which displays one or more of the properties of the protein of the invention. The probes may also be used to detect a nucleic acid encoding a SMAK protein of the invention in tissues and cells.

The SMAK protein is involved in cell apoptosis and actin stress fiber dissolution. Accordingly, in one embodiment the present invention provides a method of modulating apoptosis comprising administering an effective amount of a SMAK protein or a nucleic acid encoding a SMAK protein to a cell or animal in need thereof.

JNK and caspases are associated with apoptosis. Stress inducing agents as well as apoptotic triggers have been demonstrated to signal through stress-activated pathways leading to activation of JNK. The induction of apoptosis involves a proteolytic activation cascade of a family of cysteine proteases called caspases. The SMAK protein appears to be associated with apoptosis and accordingly may play a role in preventing neoplasia development, lymphoproliferative conditions, inflammation, and autoimmune disease. In such embodiments, it may not be desirable to inhibit the expression of the SMAK protein. Accordingly, the present invention provides a method of inhibiting or reducing cell proliferation comprising administering to an undesirable cell or to an animal in need thereof, an effective amount of an agent that promotes the expression or activity of the SMAK protein. Agents include those that may promote the SMAK protein promoter sequences to upregulate a portion of a nucleic acid sequence encoding the SMAK protein.

Under conditions where apoptotic responses are detrimental, such as ischemia and stroke, and where SMAK activity would be increased, it may be desirable to inhibit SMAK activity or expression. Accordingly, the present invention provides a method of inhibiting or reducing SMAK activity comprising administering to a cell or animal in need thereof an effective amount of an agent that inhibits the expression or activity of the SMAK protein. Agents include those that may inhibit the SMAK protein promoter sequences to downregulate a portion of a nucleic acid sequence encoding the SMAK protein.

The invention still further provides a method for identifying a substance which is capable of binding to a SMAK protein or an activated form thereof, comprising reacting the SMAK protein, or an activated form thereof, with at least one substance which can potentially bind with the SMAK protein or an activated form thereof, under conditions which permit the formation of complexes between the substance and the SMAK protein or an activated form thereof, and assaying for complexes, for free substance, for non-complexed SMAK protein or an activated form thereof, or for activation of SMAK. Specifically, a yeast two hybrid assay system may be utilized as a method for identifying proteins which interact with the protein (Fields, S. and Song, O. 1989, Nature, 34:245-247).

Still further, the invention provides a method for assaying a medium for the presence of an agonist or antagonist of the interaction of a SMAK protein or an activated form thereof, and a substance which binds to the SMAK protein or an activated form thereof. In an embodiment, the method comprises providing a known concentration of a SMAK protein, with a substance which is capable of binding to the SMAK protein and a

suspected agonist or antagonist substance under conditions which permit the formation of complexes between the substance and SMAK protein, and assaying for complexes, for free substance, for non-complexed SMAK protein, or for activation of SMAK protein.

Substances which affect the SMAK protein may also be identified using the methods of the invention by comparing the pattern and level of expression of the SMAK protein of the invention in tissues and cells in the presence, and in the absence of the substance.

Further, the present invention provides a method of identifying agents for treatment of neoplasia, lymphoproliferative conditions, arthritis, inflammation, autoimmune diseases, apoptosis, muscle atrophy, cardiomyopathy, and the like, that are related to SMAK signal transduction and actin reorganizing pathways.

The antibodies to the SMAK protein or antisense oligonucleotides complimentary to a nucleic acid encoding the SMAK protein as well as substances identified using the method of the invention may be used in the treatment of conditions involving apoptosis and actin stress fiber dissolution, preferably arthritis, apoptosis, muscle atrophy, and cardiomyopathy. Accordingly, the substances may be formulated into pharmaceutical compositions for adminstration to individuals suffering from such afflictions.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the cDNA sequence of SMAK.

Figure 2 shows the deduced amino acid sequence of SMAK.

Figure 3A shows the sequence alignment of SMAK with that of the Ste20-related kinases human SLK (hSLK), LOK and MST.

Figure 3B shows the sequence alignment of SMAK with Rat SLK, LOK, M-NAP and AT1-46.

Figure 3C shows the schematic representation of SMAK and similarity indices (%) to various other polypeptide. The catalytic domain (black) is most similar to LOK and MST1/2, two Ste20-like kinases. SMAK is highly homologous to microtubule and nuclear associated protein (M-NAP; grey) and to AT1-46 (white) in the carboxy terminal region. Numbers in brackets represent SMAK1 amino acid residues.

Figure 4A shows the Northern blot analysis of various tissue analyzed for SMAK expression and normalized for PGK-1 mRNA levels.

Figure 4B shows the Northern blot analysis of various tissue analyzed for

SMAK expression and normalized for b-actin mRNA levels.

Figure 4C shows the Northern blot analysis of SMAK mRNA expression in murine cell lines.

Figure 4D shows the Northern blot analysis of SMAK expression in various human cell lines.

Figure 5A shows expression of SMAK during C2C12 cellular differentiation.

Figure 5B shows expression of SMAK during serum stimulation of NIH3T3 fibroblasts.

Figure 6A shows an immunoblot of in vitro kinase assay of purified or immunoprecipitated SMAK 1.

Figure 6B shows inactivation of SMAK by calf intestinal alkaline phosphatase (CIAP) treatment.

Figure 7A shows immunoprecipitation and in vitro kinase assay of transfected Myc-SMAK.

Figure 7B shows immunoblot analysis illustrating that the cultures of Figure 7A expressed equivalent amounts of transfected SMAK protein.

Figure 7C shows the activation of JNK1 by SMAK in 293 cells.

Figure 8A shows immunolocalization of SMAK in C2C12 myoblasts.

Figure 8B shows the photomicrographs of Figure 8A at 400X.

Figure 9A shows induction of apoptosis in SMAK Transfected C2C12 Myoblasts.

Figure 9B shows induction of apoptosis in SMAK Transfected C2C12 Myoblasts.

Figures 9C and 9D shows double immunofluorescent staining of Myc-SMAK and annexin V-FITC in transfected C2C12 cells. Arrows denote identical cells in Figures 9C and 9D.

Figures 9E and 9F show immunodetection of the Myc-SMAKK63R lacking functional kinase activity in transfected C2C12 cells and co-localization of annexin V-FITC. Arrows denote identical cells in Figures 9E and 9F.

Figures 9G and 9H shows immunodetection of Myc-SMAK expression in cells undergoing DNA fragmentation as detected by TUNEL labeling. Arrows denote identical cells in Figures 9G and 9H.

Figure 10 shows Gal4-Luciferase reporter and Gal4-Elk1, Gal4-Jun and Gal4-CREB fusions with or without SMAK transiently transfected into 293 cells.

Figures 11A and 11B shows expression of Myc-SMAK in transiently transfected C2C12 cells inducing loss of actin stress fibers as detected by phalloidin staining. Arrow denotes the identical cell in Figures 10A and 10B.

Figures 11C and 11D show expression of Myc-RacV12 in C2C12 cells

illustrating similarly induced loss of stress fibers as evidenced by phalloidin staining. Arrow denotes the identical cell in Figures 11C and 11D.

Figures 11E and 11F show expression of transfected Myc-p65PAK illustrating induced loss of actin stress fibers. Arrow denotes the identical cell in Figures 11E and 11F.

Figure 12 shows the colocalization of SMAK, PAK, and RhoA in transfected C2C12 myoblasts.

Figures 12A, 12B, 12C, and 12D show immunodetection of transfected Myc-tagged SMAK, PAK, RhoAN19 and Rac1V12, respectively with antibody 9E10.

Figure 12E shows induction of cell death by activated RhoA in C2C12 cells. RhoAV14 expression was detected using 9E10 16 hours following transfection.

Figure 12F shows a phase contrast photomicrograph of Figure 12E.

Figures 12G and 12H show double immunodetection of transfected HA-tagged SMAK with a rabbit anti-HA antibody and of transfected Myc-RhoAN19 expressing cells using 9E10.

Figure 12I illustrates a superposition of Figures 12G and 12H showing colocalization of SMAK and RhoA.

Figures 12J, 12K, and 12L illustrate double immunodetection of transfected HA-tagged SMAK with a rabbit anti-HA antibody and of transfected Myc-PAK expressing cells using 9E10.

Figure 12L shows a superposition of Figures 12J and 12L showing colocalization of SMAK and PAK.

Figure 13 shows the schematic representation of the plasmid expression vectors used in this study. The Ste20 kinase domain (black), M-NAP (grey) and ATH (white) domains are indicated. SMAK amino acid residues are indicated at the N- and C-termini.

Figures 14A and 14B show immunodetection and phalloidin staining of Myc-SMAKΔC-expressing C2C12 cells 16 hours post-transfection.

Figures 14C and 14D show that overexpression of the Myc-SMAKΔCK63R mutant did not result in any morphological changes in C2C12 cells.

Figures 14E and 14F show forced expression of Myc-SMAKΔN induced stress fiber dissolution in overexpressing cells as shown by phalloidin staining.

Figures 14G and 14H show transfection of the AT1-46 domain (pXh2973; H and I) resulted in a loss of strongly staining actin stress fibers. Photomicrographs are shown at 400X.

Figure 14I shows enhanced apoptotic response by SMAKΔC overexpression.

Figure 14J shows the indicated expression vectors were transfected into 293T cells, immunoprecipitated using 9E10 antibodies and assayed for kinase activity on myelin basic protein MBP arrow. Equivalent aliquots of protein were also subjected to Western blot

analysis for normalization (upper panel).

Figure 15A shows caspase-3 cleavage of in vitro translated wildtype (WT) and caspase-3 cleavage site mutant (D436N) SMAK proteins.

Figure 15B shows apoptosis-induced cleavage of endogenous SMAK protein in stimulated Rat1-Myc/ER cells.

Figure 15C shows N-terminal-specific antibodies identifying the 60 kDa fragment as the kinase domain in induced Rat1-Myc/ER cells and NIH3T3 cells exposed to apoptotic triggers.

Figure 15D shows in vitro caspase-3/kinase assay on immunoprecipitated Myc-SMAK proteins.

Figure 15E shows anti-Myc tag western blot analysis showing expression of all Myc-SMAK proteins in transfected 293 cells.

DETAILED DESCRIPTION OF THE INVENTION

The following standard abbreviations for the amino acid residues are used throughout the specification: A, Ala - alanine; C, Cys - cysteine; D, Asp- aspartic acid; E, Glu - glutamic acid; F, Phe - phenylalanine; G, Gly - glycine; H, His - histidine; I, Ile - isoleucine; K, Lys - lysine; L, Leu - leucine; M, Met - methionine; N, Asn - asparagine; P, Pro - proline; Q, Gln - glutamine; R, Arg - arginine; S, Ser - serine; T, Thr - threonine; V, Val - valine; W, Trp- tryptophan; Y, Tyr - tyrosine; and p.Y., P.Tyr - phosphotyrosine, P, Ser - phosphoserine.

I. Nucleic Acid Molecules Encoding SMAK

As hereinbefore mentioned, the invention provides an isolated and purified nucleic acid molecule having a sequence encoding a SMAK protein. The term "isolated and purified" refers to a nucleic acid substantially free of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors, or other chemicals when chemically synthesized. The term "nucleic acid" is intended to include DNA and RNA and can be either double stranded or single stranded.

The nucleic acid sequence of the cDNA encoding the SMAK protein is shown in Figure 1.

It will be appreciated that the invention includes nucleic acid molecules encoding truncations of SMAK protein, and analogs and homologs of SMAK and truncations thereof, as described herein. It will further be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention.

Another aspect of the invention provides a nucleic acid molecule which hybridizes under high stringency conditions to a nucleic acid molecule which comprises a sequence which encodes SMAK protein having the amino acid sequence shown in Figure 2. Appropriate stringency conditions which promote DNA hybridization are known to those

skilled in the art, or can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. For example, 6.0 x sodium chloride/sodium citrate (SSC) at about 45°C, followed by a wash of 2.0 x SSC at 50°C may be employed. The stringency may be selected based on the conditions used in the wash step. By way of example, the salt concentration in the wash step can be selected from a high stringency of about 0.2 x SSC at 50°C. In addition, the temperature in the wash step can be at high stringency conditions, at about 65°C.

Isolated and purified nucleic acid molecules encoding a protein having the activity of SMAK protein as described herein, and having a sequence which differs from the nucleic acid sequence shown in Figure 1, due to degeneracy in the genetic code are also within the scope of the invention.

In addition, DNA sequence polymorphisms within the nucleotide sequence of the SMAK protein (especially those within the third base of a codon) may result in "silent" mutations in the DNA which do not affect the amino acid encoded. However, DNA sequence polymorphisms may lead to changes in the amino acid sequences of SMAK protein within a population. It will be appreciated by one skilled in the art that these variations in one or more nucleotides of the nucleic acids encoding proteins having the activity of the SMAK protein may exist among individuals within a population due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of the invention.

An isolated and purified nucleic acid molecule of the invention which comprises DNA can be isolated by preparing a labelled nucleic acid probe based on all or part of the nucleic acid sequence shown in Figure 1 and using this labelled nucleic acid probe to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). For instance, a cDNA library made from human cells such as B cells can be used to isolate a cDNA encoding a protein having SMAK activity by screening the library with the labelled probe using standard techniques. Alternatively, a genomic DNA library can be similarly screened to isolate a genomic clone encompassing a gene encoding a SMAK protein. Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

An isolated and purified nucleic acid molecule of the invention which is DNA can also be isolated by selectively amplifying a nucleic acid encoding a SMAK protein using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence shown in Figure 1. A nucleic acid can be amplified from cDNA or genomic DNA using these oligonucleotide primers and standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. It will be appreciated that cDNA may be prepared from mRNA, by isolating

total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., Biochemistry, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

An isolated and purified nucleic acid molecule of the invention which is RNA can be isolated by cloning a cDNA encoding a SMAK protein into an appropriate vector which allows for transcription of the cDNA to produce an RNA molecule which encodes a protein which exhibits SMAK protein activity. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector, cDNA can be transcribed in vitro with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

A nucleic acid molecule of the invention may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polydeoxynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,066; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

Determination of whether a particular nucleic acid molecule encodes a protein having SMAK protein activity can be accomplished by expressing the cDNA in an appropriate host cell by standard techniques, and testing the ability of the expressed protein to undergo caspase-3 cleavage and mediate apoptosis and actin stress fibre dissolution. A cDNA having the biological activity of a SMAK protein so isolated can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

The sequence of a nucleic acid molecule of the invention may be inverted relative to its normal presentation for transcription to produce an antisense nucleic acid molecule. An antisense nucleic acid molecule may be constructed using chemical synthesis and enzymatic ligation reactions using procedures known in the art.

II. <u>SMAK Proteins</u>

As hereinbefore mentioned, the present inventors have isolated and characterized a novel caspase-3 activated protein kinase, designated SMAK. SMAK is highly related to LOK, an Ste20-related protein kinase preferentially expressed in lymphocytes in that it shares about a 70% homology in the kinase domain. SMAK also shares extensive homology to microtubule and nuclear associated protein (M-NAP) amounting to about 65% homology and to AT1-46 with a homology of about 60%. The SMAK

cDNA encodes a protein with a predicted molecular weight of 148 kDa. The amino acid sequence of the SMAK protein is shown in Figure 2.

The inventors have shown that the SMAK protein contains an SH3-binding motif and a coiled-coil structure in the C-terminal region and an N-terminal serine/threonine kinase catalytic domain with the signature sequence Gly-X-Gly-X-X-Gly.

SMAK mRNA is widely expressed in a variety of tissues including skeletal muscle, heart, thymus, brain, colon, spleen, lung, kidney, testes, uterus, and liver. The SMAK protein is not expressed in undifferentiated P19 embryo carinoma cells which suggests that SMAK has more of a role in differentiated cell types.

The proteins of the present invention include truncations of the SMAK protein, and analogs, and homologs and truncations thereof as described herein.

The truncated proteins may have an amino group (-NH2), a hydrophobic group (for example, carbobenzoxyl, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl (PMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the amino terminal end. The truncated proteins may have a carboxyl group, an amido group, a T-butyloxycarbonyl group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of the SMAK protein as shown in Figure 2 or truncations thereof as described herein, which may include, but are not limited to one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the SMAK amino acid sequence with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to the SMAK protein. Non-conserved substitutions involve replacing one or more amino acids of the SMAK amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the SMAK protein. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length.

The proteins of the invention also include homologs of the SMAK protein (Figure 2) and/or truncations thereof as described herein. Such homologs are proteins whose amino acid sequences are comprised of the amino acid sequences of SMAK protein regions from other species that hybridize under stringent hybridization conditions with a probe used to obtain the SMAK protein.

The invention also contemplates isoforms of the protein of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention or

subtle spliced differences which alter the overall number of amino acids resulting in a slight shift in molecular weight. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein. The inventors have demonstrated that the SMAK mRNA is expressed and exists in at least three distinct isoforms, all of which are expressed at similar levels in all tissues tested with the exception of the testes and colon tissues.

The present invention also includes SMAK protein conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion proteins. Further, the present invention also includes activated or phosphorylated SMAK proteins of the invention. Additionally, immunogenic portions of SMAK proteins are within the scope of the invention.

SMAK proteins of the invention may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention having a sequence which encodes a SMAK protein of the invention may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses (e.g. replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression vectors are "suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid molecule. Operatively linked is intended to mean that the nucleic acid is linked to regulatory sequences in a manner which allows expression of the nucleic acid.

The invention therefore contemplates a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary regulatory sequences for the transcription and translation of the inserted protein-sequence. Suitable regulatory sequences may be derived from a variety of sources, including mammalian, bacterial, fungal, viral or insect genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory sequences is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the

native SMAK gene and/or its flanking regions.

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression, by transcription of the DNA molecule, of an RNA molecule which is antisense to the nucleotide sequence shown in Figure 1. Regulatory sequences operatively linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance a viral promoter and/or enhancer, or regulatory sequences can be chosen which direct tissue or cell type specific expression of antisense RNA.

The recombinant expression vectors of the invention may also contain a selectable marker gene which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, b-galactosidase, chloramphenicol acetyltransferase, firefly luciferase, green fluorescent protein, alkaline phosphatase, yellow fluorescent protein, blue fluorescent protein or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as b-galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of the target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Typical fusion expression vectors include pGEX (Pharmacia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the recombinant protein.

Recombinant expression vectors can be introduced into host cells to produce a transformant host cell. The term "transformant host cell" is intended to include

prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. The terms "transformed with", "transfected with", "transformation" and "transfection" are intended to encompass introduction of nucleic acid (e.g. a vector) into a cell by one of many possible techniques known in the art. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the SMAK proteins of the invention may be expressed in mammalian cells, bacterial cells such as E. coli, insect cells (using baculovirus) or yeast cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

Mammalian cells suitable for carrying out the present invention include, among others: Cos-7 (green monkey kidney), Saos-2 (human osteosarcoma), PC12, NIH-3T3, MONC, SY5Y, P19, G361 (human melanoma), A549 (human lung carcinoma), SW480 (human colorectal adenocarcinoma), Raji (human Burkitt's lymphoma), MOLT-4 (human lymphoblastic leukemia), K562 (human chronic myelogenous leukemia), S3 (Hela cells), HL-60 (human promyelocytic leukemia, and breast neoplasia cell lines; T47Ds, MCF-7s and C127s, COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter (e.g., derived from viral material such as polyoma, Adenovirus 2, cytomegalovirus, retrovirus (pBabe and LXHSD), and Simian Virus 40), as well as other transcriptional and translational control sequences. Examples of mammalian expression vectors include pCDM8 (Seed, B., (1987) Nature 329:840) and pMT2PC (Kaufman et al. (1987), EMBOJ. 6:187-195).

Bacterial host cells suitable for carrying out the present invention include E. coli, B. subtilis, Salmonella typhimurium, and various species within the genus' Pseudomonas, Streptomyces, and Staphylococcus, as well as many other bacterial species well known to one of ordinary skill in the art. Suitable bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the b-lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the trp promoter (Nichols and Yanofsky, Meth in Enzymology

101:155, 1983) and the tac promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Suitable expression vectors include but are not limited to bacteriophages such as lambda derivatives or plasmids such as pBR322 (see Bolivar et al., Gene 2:9S, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.). Typical fusion expression vectors which may be used are discussed above, e.g. pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ). Examples of inducible non-fusion expression vectors include pTrc (Amann et al., (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89).

Yeast and fungi host cells suitable for carrying out the present invention include, but are not limited to Saccharomyces cerevisae, the genera Pichia or Kluyveromyces and various species of the genus Aspergillus. Examples of vectors for expression in yeast S. cerivisae include pYepSec1 (Baldari. et al., (1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz, (1982) Cell 30:933-943), pJRY88 (Schultz et al., (1987) Gene 54:113-123), and pYES2 (Invitrogen Corporation, San Diego, CA). Protocols for the transformation of yeast and fungi are well known to those of ordinary skill in the art (see Hinnen et al., PNAS USA 75:1929, 1978; Itoh et al., J. Bacteriology 153:163, 1983, and Cullen et al. (Bio/Technology 5:369, 1987).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, the proteins of the invention may be expressed from plant cells (see Sinkar et al., J. Biosci (Bangalore) 11:47-58, 1987, which reviews the use of Agrobacterium rhizogenes vectors; see also Zambryski et al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of expression vectors for plant cells, including, among others, pAS2022, pAS2023, and pAS2034).

Insect cells suitable for carrying out the present invention include cells and cell lines from Bombyx or Spodotera species. Baculovirus vectors available for expression of proteins in cultured insect cells (SF 9, SF 21, and T.ni-High Five cells) include the pAc series (Smith et al., (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow, V.A., and Summers, M.D., (1989) Virology 170:31-39) and pBAC PAK.

Alternatively, the proteins of the invention may also be expressed in non-human transgenic animals such as mice, rats, rabbits, sheep and pigs (see Hammer et al.

(Nature 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866).

The invention further provides a recombinant expression vector for the transcription and translation in invertebrate animals including, but not limited to, zebrafish, xenopus and drosophila.

The proteins of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

N-terminal or C-terminal fusion proteins comprising the SMAK protein of the invention conjugated with other molecules, such as proteins may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of the protein, and the sequence of a selected protein or selectable marker protein with a desired biological function. The resultant fusion proteins contain the SMAK protein fused to the selected protein or marker protein as described herein. Examples of proteins which may be used to prepare fusion proteins include green fluorescent protein (GFP), yellow fluorescent protein, blue fluorescent protein, alkaline phosphatase, immunoglobulins, glutathione-S-transferase (GST), hemagglutinin (HA), and truncated myc.

Phosphorylated or activated SMAK proteins of the invention may be prepared using the method described in Reedijk et al. The EMBO Journal 11(4):1365, 1992.

III. <u>Utility of the Nucleic Acid Molecules and Proteins of the Invention</u>

<u>Diagnostic Uses</u>

The nucleic acid molecules of the invention allow those skilled in the art to construct nucleotide probes for use in the detection of nucleic acid sequences in biological materials. Suitable probes include nucleic acid molecules based on nucleic acid sequences encoding at least 6 sequential amino acids from regions of the SMAK protein as shown in Figure 2. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as 32P, 3H, 14C or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and luminescent compounds. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleotide to be detected and the amount of nucleotide available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory Manual (2nd ed.). The nucleic acid probes may be used to detect

genes, preferably in human cells, that encode the SMAK protein. The nucleotide probes may be useful in the diagnosis of disorders of cell transformation such as muscle atrophy. The nucleotide probes may also be used as a diagnostic tool on tissue biopsies to assess the transformed state of the cell. The probes may also be used in in situ hybridization of early embryos to assess both the onset and pattern of expression during development.

SMAK proteins of the invention can be used to prepare antibodies specific for the SMAK proteins that may be used to detect the SMAK protein in a biological sample. Antibodies can be prepared which bind a distinct epitope in an unconserved region of the protein. An unconserved region of the protein is one which does not have substantial sequence homology to other proteins.

Conventional methods can be used to prepare the antibodies. For example, by using a peptide of the SMAK protein, polyclonal antisera or monoclonal antibodies can be made using standard methods. A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of the peptide which elicits an antibody response in the mammal. Techniques for conferring immunogenicity on a peptide include conjugation to carriers or other techniques well known in the art. For example, the protein or peptide can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay procedures can be used with the immunogen as antigen to assess the levels of antibodies. Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. The preparation of anti-SMAK antibodies is described in Example 1.

To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding hybridoma cells. Such techniques are well known in the art, (e.g., the hybridoma technique originally developed by Kohler and Milstein (Nature 256, 495-497 (1975)) as well as other techniques such as the human B-cell hybridoma technique (Kozbor et al., Immunol. Today 4, 72 (1983)), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. Monoclonal Antibodies in Cancer Therapy (1985) Allen R. Bliss, Inc., pages 77-96), and screening of combinatorial antibody libraries (Huse et al., Science 246, 1275 (1989)). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the peptide and the monoclonal antibodies can be isolated. Therefore, the invention also contemplates hybridoma cells secreting monoclonal antibodies with specificity for the SMAK protein as described herein.

The term "antibody" as used herein is intended to include fragments thereof which also specifically react with a SMAK protein, or peptide thereof, having the activity of the SMAK protein. Antibodies can be fragmented using conventional techniques

and the fragments screened for utility in the same manner as described above. For example, F(ab')2 fragments can be generated by treating antibody with pepsin. The resulting F(ab')2 fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods may be used to make chimeric antibodies containing the immunoglobulin variable region which recognizes the gene product of SMAK antigens of the invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive with a protein of the invention as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules may be made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, reactive against proteins of the invention may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with peptides produced from the nucleic acid molecules of the present invention. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies, or fragments thereof.

Antibodies specifically reactive with the SMAK protein, or derivatives thereof, such as enzyme conjugates or labeled derivatives, may be used to detect the SMAK protein in various biological materials, for example they may be used in any known immunoassays which rely on the binding interaction between an antigenic determinant of

the SMAK protein, and the antibodies. Examples of such assays are radioimmunoassays, enzyme immunoassays (e.g. ELISA), immunofluorescence, immunoprecipitation, latex agglutination, hemagglutination, and histochemical tests. Thus, the antibodies may be used to detect and quantify the SMAK protein in a sample in order to determine its role in particular cellular events or pathological states, and to diagnose and treat such pathological states.

In particular, the antibodies of the invention may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect the SMAK protein, to localise it to particular cells and tissues and to specific subcellular locations, and to quantitate the level of expression.

Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect the SMAK protein. Generally, an antibody of the invention may be labelled with a detectable substance and the SMAK protein may be localised in tissue based upon the presence of the detectable substance. Examples of detectable substances include various enzymes, fluorescent materials, luminescent materials and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline phosphatase, b-galactosidase, or acetylcholinesterase; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; and examples of suitable radioactive material include radioactive iodine I125, I131 or tritium. Antibodies may also be coupled to electron dense substances, such as ferritin or colloidal gold, which are readily visualised by electron microscopy.

Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the SMAK protein. By way of example, if the antibody having specificity against the SMAK protein is a rabbit IgG antibody, the second antibody may be goat anti-rabbit gamma-globulin labelled with a detectable substance as described herein.

Where a radioactive label is used as a detectable substance, the SMAK protein may be localized by autoradiography. The results of autoradiography may be quantitated by determining the density of particles in the autoradiographs by various optical methods, or by counting the grains.

As discussed herein, the SMAK protein likely plays a role in apoptosis and actin stress fiber dissolution in cells. Therefore, the above described methods for detecting nucleic acid molecules and SMAK proteins of the invention, can be used to monitor cell death. It would also be apparent to one skilled in the art that the above described methods may be used to study the expression of the SMAK protein and, accordingly, will provide

further insight into the role of the SMAK protein in cells.

Therapeutic Uses

The SMAK protein of the invention is likely involved in the regulation of cell signalling pathways that control cell death. Accordingly, the present invention provides a method of modulating cell death or apoptosis comprising administering an effective amount of a SMAK protein or a nucleic acid encoding a SMAK protein to a cell or animal in need thereof. The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired results.

In another aspect the present invention provides a method of modulation of cell proliferation. In one embodiment, the invention provides a method of inhibiting or reducing cell proliferation, such as in neoplasia, by administering to a cell or animal an effective amount of an agent that promotes the expression or the biological activity of the SMAK protein.

In another embodiment, the present invention provides a method of inducing cell proliferation by administering to a cell or an animal an effective amount of an agent that inhibits the expression or the biological activity of the SMAK protein. Agents that inhibit the activity of the SMAK protein include antibodies to SMAK protein. Agents that inhibit the expression of the SMAK gene include antisense oligonucleotides to a SMAK nucleic acid sequence. Both of these are described above.

In addition to antibodies and antisense oligonucleotides, other substances that inhibit SMAK protein expression or activity may also be identified. Substances which affect SMAK protein activity can be identified based on their ability to bind to the SMAK protein. Therefore, the invention also provides methods for identifying substances which are capable of binding to the SMAK protein. In particular, the methods may be used to identify substances which are capable of binding to, and in some cases activating (i.e., phosphorylating) and in other cases deactivating the SMAK protein of the invention.

Substances which can bind with the SMAK protein of the invention may be identified by reacting the SMAK protein with a substance which potentially binds to the SMAK protein, and assaying for complexes, for free substance, or for non-complexed SMAK protein, or for activation of the SMAK protein. In particular, a yeast two hybrid assay system may be used to identify proteins which interact with the SMAK protein (Fields, S. and Song, O., 1989, Nature, 340:245-247).

Conditions which permit the formation of substance and SMAK protein complexes may be selected having regard to factors such as the nature and amounts of the substance and the protein.

The substance-protein complex, free substance or non-complexed proteins may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel

electrophoresis, agglutination, or combinations thereof. To facilitate the assay of the components, antibody against the SMAK protein or the substance, or labelled SMAK protein, or a labelled substance may be utilized. The antibodies, proteins, or substances may be labelled with a detectable substance as described above.

Substances which bind to and activate the SMAK protein of the invention may be identified by assaying for phosphorylation of the tyrosine residues of the protein.

Substances which bind to and inactivate the SMAK protein of the invention may be identified by assaying for reduction in phosphorylation of the fully activated protein.

The SMAK protein, or the substance used in the method of the invention may be insolubilized. For example, the SMAK protein or substance may be bound to a suitable carrier. Examples of suitable carriers are agarose, cellulose, dextran, Sephadex, Sepharose, carboxymethyl cellulose polystyrene, filter paper, ion-exchange resin, plastic film, plastic tube, glass beads, polyamine-methyl vinyl-ether-maleic acid copolymer, amino acid copolymer, ethylene-maleic acid copolymer, nylon, silk, etc. The carrier may be in the shape of, for example, a tube, test plate, beads, disc, sphere etc.

The insolubilized protein or substance may be prepared by reacting the material with a suitable insoluble carrier using known chemical or physical methods, for example, cyanogen bromide coupling.

The proteins or substance may also be expressed on the surface of a cell using the methods described herein.

The invention also contemplates a method for assaying for an agonist or antagonist of the binding of the SMAK protein with a substance which is capable of binding with the SMAK protein. The agonist or antagonist may be an endogenous physiological substance or it may be a natural or synthetic substance. Substances which are capable of binding with the SMAK protein may be identified using the methods set forth herein.

It will be understood that the agonists and antagonists that can be assayed using the methods of the invention may act on one or more of the binding sites on the protein or substance including agonist binding sites, competitive antagonist binding sites, non-competitive antagonist binding sites or allosteric sites.

The invention also makes it possible to screen for antagonists that inhibit the effects of an agonist of the interaction of the SMAK protein with a substance which is capable of binding to the SMAK protein. Thus, the invention may be used to assay for a substance that competes for the same binding site of the SMAK protein.

The methods described above may be used to identify a substance which is capable of binding to an activated SMAK protein, and to assay for an agonist or antagonist of the binding of activated SMAK protein, with a substance which is capable of binding with activated SMAK protein. An activated (i.e. phosphorylated) the SMAK protein may

be prepared using the methods described (for example in Reedijk et al. The EMBO Journal, 11(4):1365, 1992) for producing a tyrosine phosphorylated protein.

It will also be appreciated that intracellular substances which are capable of binding to the SMAK protein may be identified using the methods described herein.

The invention further provides a method for assaying for a substance that affects an SMAK protein regulatory pathway comprising administering to a non-human animal or to a cell, or a tissue of an animal, a substance suspected of affecting a SMAK protein regulatory pathway, and quantitating the SMAK protein or nucleic acids encoding the SMAK protein, or examining the pattern and/or level of expression of SMAK protein, in the non-human animal or tissue, or cell. SMAK protein may be quantitated and its expression may be examined using the methods described herein.

The substances identified by the methods described herein, may be used for modulating SMAK protein regulatory pathways and accordingly may be used in the treatment of conditions involving perturbation of SMAK protein signalling pathways. In particular, the substances may be particularly useful in the treatment of disorders of cell death.

As stated previously, SMAK protein may be involved in cell proliferation and inhibitors of the SMAK protein may be useful in modulating disorders involving cell proliferation such as neoplasia. In contrast, stimulators of the SMAK protein may be useful in the modulation of disorders requiring reduction of proliferation. Accordingly, substances that stimulate the SMAK protein (for example, identified using the methods of the invention) may be used to stimulate cell death or apoptosis. Substances which stimulate apoptosis may be useful in the treatment of cancer.

Peptide Mimetics

The present invention also include peptide mimetics of the SMAK protein of the invention. For example, a peptide derived from a binding domain of SMAK will interact directly or indirectly with an associated molecule in such a way as to mimic the native binding domain. Such peptides may include competitive inhibitors, enhancers, peptide mimetics, and the like. All of these peptides as well as molecules substantially homologous, complementary or otherwise functionally or structurally equivalent to these peptides may be used for purposes of the present invention.

"Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which may or may not contain amino acids and/or peptide bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible

sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics may be designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides of the invention may also be used to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity relationships can be used to design either modified peptides, or other small molecules or lead compounds which can be tested for predicted properties as related to the target molecule. The activity of the lead compounds can be evaluated using assays similar to those described herein.

Information about structure-activity relationships may also be obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in association with a target molecule, and the X-ray structure of the complex is determined. The structure can then be compared to the structure of the target molecule in its native state, and information from such a comparison may be used to design compounds expected to possess desired activities.

Pharmaceutical Compositions

All of the above described substances (such as the SMAK protein, the nucleic acid encoding the SMAK protein, antibodies to the SMAK protein, antisense oligonucleotides to the nucleic acid molecules and substances that modulate the SMAK protein activity) may be formulated into pharmaceutical compositions for adminstration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic

effects. The substances may be administered to living organisms including humans, and animals. Administration of a therapeutically active amount of the pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of antibody to elicit a desired response in the individual. Dosage regima may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The active substance may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active substance may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. If the active substance is a nucleic acid encoding a SMAK protein or an antisense oligonucleotide it may be delivered using techniques known in the art. Recombinant molecules comprising an antisense sequence or oligonucleotide may be directly introduced into cells or tissues in vivo using delivery vehicles such as retroviral vectors, adeno viral vectors and DNA virus vectors. They may also be introduced using physical techniques such as microinjection and electroporation or chemical methods such as co-precipitation and incorporation of DNA into liposomes.

The compositions described herein can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to subjects, such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable PH and iso-osmotic with the physiological fluids.

The reagents suitable for applying the methods of the invention to identify substances that affect the SMAK protein may be packaged into convenient kits providing the necessary materials packaged into suitable containers. The kits may also include suitable supports useful in performing the methods of the invention.

<u>Experimental Models</u>

The invention also provides methods for studying the function of the SMAK protein. Cells, tissues and non-human animals that express or over-express SMAK protein may be prepared by transfecting cells, tissues or oocytes (to prepare transgenic animals)

with a recombinant expression vector of the invention (as described previously). In particular, transgenic technology (via nuclear oocyte microinjection of naked DNA) will assay the effect of over expression or alterations of the SMAK protein expression in various developmental systems, including bone development, neurogenesis, mammary development, lung epithelial development.

Cells, tissues, and non-human animals lacking in SMAK protein expression or partially lacking in SMAK protein expression may be developed using recombinant expression vectors of the invention having specific deletion or insertion mutations in the SMAK gene. A recombinant expression vector may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a SMAK protein deficient cell, tissue or animal.

Null alleles may be generated in cells, such as embryonic stem cells by deletion mutation. A recombinant SMAK gene may also be engineered to contain an insertion mutation which inactivates the SMAK protein. Such a construct may then be introduced into a cell, such as an embryonic stem cell, by a technique such as transfection, electroporation, injection etc. Cells lacking an intact SMAK gene may then be identified, for example by Southern blotting, Northern Blotting or by assaying for SMAK protein using the methods described herein. Such cells may then be fused to embryonic stem cells to generate transgenic non-human animals deficient in SMAK protein. Germline transmission of the mutation may be achieved, for example, by aggregating the embryonic stem cells with early stage embryos, such as 8 cell embryos, in vitro; transferring the resulting blastocysts into recipient females and; generating germline transmission of the resulting aggregation chimeras. Such a mutant animal may be used to define specific cell populations, developmental patterns and <u>in vivo</u> processes, normally dependent on SMAK protein expression.

The present invention also includes the preparation of tissue specific knock-outs of the SMAK gene.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

EXAMPLE 1

SMAK, an Ste20-related kinase.

In an effort to identify protein kinases that exhibit differential expression during myoblast differentiation, a 1.6 kb partial cDNA insert encoding a mouse Ste20-like kinase was used to survey mRNA samples from differentiating myoblasts.

Cloning and Analysis of SMAK

Full length SMAK (Genbank BankIt # 242010) was obtained from an adult mouse muscle l gt11 cDNA library following plaque screening (Sambrook, J. et al., 1989)

using a partial SMAK cDNA clone obtained through two hybrid screening. Full length cDNA inserts were subcloned into pBluescript and sequenced on an ABI automated sequencer. Homology searches were performed using NCBI Blast software and homologies are presented as percent identities. The serine/threonine kinase subdomains were identified by alignment of the consensus amino acid sequence of the catalytic domain (Hanks and Hunter, 1995) to that of SMAK. Multiple alignment analysis was performed using the MegAlign program from the DNAStar software package. Further analyses for consensus protein motifs were performed using PSORT, Motif Finder and PRO Site.

Northern blot analysis revealed high level expression in myoblasts that was strongly downregulated following myotube formation (not shown). Therefore, to further characterize the mouse kinase, a muscle lambda gt11 cDNA library was screened to isolate clones containing full length cDNAs. One clone (3E5) was found to contain a 5253 bp cDNA encoding full length protein (Figure 1). Clone 3E5 encoded a 1202 amino acid polypeptide and shared over 90% nucleotide identity with sequences from the guinea pig (Itoh, et al., 1997) human and rat Ste20-like kinases (SLK) recently deposited in the database, suggesting that 3E5 represents the murine homolog. Inspection of the 3' untranslated region (UTR) did not reveal the existence of a poly-A tail or a consensus polyadenylation signal, suggesting that 3E5 bears a partial 3'UTR. However, several AU-rich motifs, previously implicated in growth factor-dependent mRNA turnover (Ross, 1996), are present in the 3'UTR.

Searches for homologous proteins revealed that the kinase catalytic domain was closely related to LOK and MST1/2, members of the Ste20 family of serine/threonine kinases. Interestingly, the central portion of 3E5 was found to be highly homologous to microtubule and nuclear associated protein (M-NAP). The remaining carboxy terminal region of the protein displayed high homology to AT1-46, a cDNA clone isolated from astrocyte mRNA. Because of an increasing number of Ste20-related kinases and to avoid potential confusion with other family members, the Ste20-like kinase encoded by clone 3E5 was renamed. Therefore, due to its relatedness to Ste20, M-NAP and AT1-46, this novel protein kinase has been termed SMAK (Figures 1 and 2). Searches for known protein motifs using PSORT revealed putative nuclear localization signals at positions 15, 422, and 1149. However, nuclear localization was never observed following transfection and immunostaining of Myc epitope-tagged SMAK (see Figure 8). A consensus SH3 binding (P-X-X-P-X) site was found at position 735 (Pawson and Scott, 1997), suggesting a potential interaction with SH3 domain-containing proteins. Other than a C-terminal region unusually rich in charged amino acids, database scans using MotifFinder failed to detect the presence of other consensus protein motifs.

Further analysis of the coding region revealed that SMAK encoded an N-terminal serine/threonine kinase catalytic domain, with the signature sequence

Gly-X-Gly-X-X-Gly identifying subdomain I at amino acid residue 41 (Figure 3A). The conserved lysine residue within the ATP binding site of subdomain II was found at amino acid residue 63. The catalytic core extended further, up to residue 282 and presented all the characteristic subdomains of serine/threonine kinases (Hanks and Hunter, 1995) (Figure 3A). Alignments and database scans revealed that SMAK displayed 74% identity, in the kinase domain, to LOK, a novel kinase preferentially expressed in lymphocytes (Kuramochi, et al., 1997) (Figure 3A). The SMAK kinase domain was also found to be related to MST1 and MST2, both Ste20-like kinases (Schinkmann and Blenis, 1997; Katoh, et al., 1995; Creasy and Chernoff, 1995; Leung et al., 1995; and Manser et al., 1995). In the kinase subdomain VIII, characterizing kinase family members, SMAK presented the Ste20 signature motif, suggesting that it represents a novel family member (Figure 3A). Further analysis showed that the central portion of SMAK, from residues 339 to 947 (Figure 2B) shared 70% identity with microtubule and nuclear associated protein (M-NAP). The remainder of SMAK, from residues 788 to 926, overlapping slightly with the M-NAP domain, displayed 63% identity to AT1-46 (Schaar, et al., 1996) (Figure 3B). Interestingly, M-NAP and AT1-46 share significant homology in the overlapping region. The function of both M-NAP and AT1-46 proteins is currently unknown. A second region of homology (71%) to AT1-46 was observed from SMAK residues 957 to 1171. Interestingly, these two AT1-46 domains were found to be 56% identical to the C-terminal region of LOK which also shares extensive identity to AT1-46 in its C-terminus (Figure 3B). These observations suggest that SMAK and LOK may represent members of a new protein kinase family. Furthermore, the AT1-46 homology domain might be a novel protein motif likely to be important for LOK and SMAK functions. This new motif has been termed the ATH domain, for AT1-46 homology domain (see Figure 3C).

__Example 2__

Expression of SMAK in tissues and cell lines

To gain insights into the role of SMAK, adult human RNA samples were surveyed for SMAK expression by Northern blotting.

Cell culture and Transfections.

C2C12 cells were maintained in Dulbecco's modified Eagle medium supplemented with 15% fetal calf serum (FCS) and induced to differentiate in DMEM containing 2% horse serum. NIH3T3, P19 and 293 cells were grown in DMEM containing 10% FCS. 3T3 cells were straved in DMEM supplemented with 0.5% FCS and subsequently stimulated with growth medium where indicated. The cultures were transfected by Lipofectamine (Gibco/BRL) according to the manufacturer's instructions using 2 μg of plasmid DNA.

SMAK expression analysis.

For expression analysis, total RNA from various adult mouse tissues and cell

lines was prepared (Sambrook et al., 1989) and poly-A+ RNA was isolated by one round of selection through oligo dT cellulose (Sambrook et al., 1989). A total of 4 mg of poly-A+ RNA for each cell line was subjected to Northern blot analysis using a SMAK-specific probe. Analysis of human tissues and cell line RNA was performed by Northern hybridization of a SMAK probe to Multiple Tissue Northerns (Clontech) containing 2 μg of poly-A+ RNA per lane. Human RNA filters were washed under reduced stringency (0.2x SSC/0.1% SDS/55°C). Analysis of SMAK protein expression was performed by lysing cultures (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton-X 100 and 1 mg/ml of each aprotinin, pepstatin and leupeptin) and subjecting 20 μg of total lysate to western blot analysis using anti-SMAK rabbit polyclonal antibody (Sambrook et al., 1989). Reactive proteins were detected by ECL (Amersham) using a goat anti-rabbit horse radish peroxidase (HRP) labeled secondary antibody. SMAK polyclonal antibodies were generated by immunization of New Zealand rabbits using purified GST-SMAK95-551, encompassing part of the kinase and M-NAP domains. Specific immunoreactivity to murine SMAK protein was observed in 293 cells transfected with full length and truncated SMAK expression vectors.

For expression studies and immunolocalization experiments, HA- or Myc-tagged pcDNA3 (Invitrogen) expression vectors bearing full length, kinase dead or truncated SMAK, were constructed using standard cloning procedures (Sambrook et al., 1989). The kinase inactive mutant was generated through site directed PCR-mediated mutagenesis. The SMAKD3' truncation (residues 1-950) was obtained by removing the last 263 amino acids through XhoI restriction enzyme digestion and religation . Following transfection into C2C12, the cultures were fixed for 10 minutes in 4% paraformaldehyde and SMAK protein was detected using 9E10 monoclonal antibodies in conjunction with FITC-labeled secondary antibodies.

Analysis of poly-A+ RNA from various tissues with a SMAK-specific probe revealed the existence of at least three distinct isoforms of about 6, 7 and 8 kb (Figure 4A). All isoforms were expressed at similar levels with the exception that testes and colon tissues were found to express relatively higher levels of the 5 and 6 kb isoforms. Analysis of mouse tissues, including heart, skeletal muscle and brain, showed a similar pattern of expression (not shown). RNA analysis of mouse cell lines showed that SMAK was not expressed in undifferentiated P19 embryocarcinoma cells (Figure 4B), suggesting a role for SMAK in more differentiated cell types. In contrast to tissue samples which predominantly expressed the 7 kb mRNA (Figure 3C), the other murine cell lines surveyed displayed similar levels of all three isoforms (Figure 4B).

Analysis of human tumor cell lines showed that the chronic myelogenous leukemia cell line K-562 and the colon adenocarcinoma cell line SW480 expressed high levels of SMAK mRNA, relative to normal blood lymphocytes and colon tissue,

respectively (Figure 4C). Whether high levels of SMAK correlates with these specific tumor phenotypes remains to be clarified. Other human tumor cell lines were found to express SMAK at levels that were comparable to normal human tissues of the same origin.

To facilitate the biochemical characterization and to gain insights into SMAK functions, anti-SMAK antibodies were developed. Rabbit polyclonal antibodies were generated against a GST-SMAK fusion protein encompassing residues 95 to 551 and used in western blot analysis of C2C12 cultures. Specific reactivity to murine SMAK protein was observed at approximately 220 and 170 kDa in control 293 cells expressing full length SMAK or a C-terminal truncation (SMAKD3'), respectively (Figure 5A). No signal was detected in control vector transfected 293 cells. The p220 SMAK is relatively larger than the predicted molecular weight of 148 kDa, suggesting that SMAK is subject to post-translational modifications (Figure 5A). In contrast to Northern blotting, Western analysis of C2C12 myoblast cultures revealed 6 SMAK immunoreactive polypeptides (Figure 5A). Some of these may represent breakdown products or, alternatively, they might reflect post-translational modifications. Interestingly, SMAK protein expression decreased as myogenic differentiation progressed. A marked reduction was observed 3 days following the onset of differentiation, well after the majority of the myoblasts have fused into multinucleated myotubes (not shown). Alternatively, the observed decrease in SMAK expression could be due to growth factor depletion which was used to initiate differentiation. Furthermore, the 3' UTR of cDNA clone 3E5 displays several AU-rich motifs potentially mediating growth factor-dependent stability of SMAK mRNA. To test whether the downregulation of SMAK during C2C12 differentiation could be attributed to growth factor depletion, NIH 3T3 cells were serum-starved for 24 hours, stimulated with 10% serum and analyzed for SMAK expression by western blotting. As shown in Figure 4B, serum starvation of 3T3 cells resulted in a rapid decrease in SMAK expression. Following the stimulation of 3T3 starved cultures for 24 hours, a SMAK reactive species of approximately 140 kDa was induced, likely representing one of several isoforms (Figure 5B). In contrast to C2C12, 3T3 cells were found to express only 3 SMAK protein isoforms. With the exception of p220 SMAK, these isoforms did not correspond to any of the ones detected in C2C12 cells, suggesting differential splicing or processing among cell types.

Example 3

Cellular Distribution of SMAK

Because our anti-SMAK antibodies failed to detect antigen in fixed cells, a Myc epitope tag expression vector carrying full length SMAK was constructed and transfected into C2C12 myoblasts. To evaluate the cellular distribution of SMAK, immunostaining was performed using 9E10 monoclonal antibodies on fixed cultures following transient transfection. Interestingly, 9E10-positive cells displayed high concentration of Myc-SMAK protein in distinct cytosolic domains, predominantly at the periphery of the

cells (Figure 8A).The distribution of these domains appeared suggestive of scaffold structures such as growth factor receptors or focal adhesion complex from which SMAK signaling could occur. Transfection of the kinase dead mutant SMAKK63R did not alter the cellular distribution (Figure 8B).

Example 4

Modulation of SMAK activity by extracellular stimuli

As shown in Figure 6A, bacterially expressed GST-SMAK and immunoprecipitated Myc-SMAK autophosphorylated and efficiently phosphorylated MBP and histone H1 in vitro. However, SMAK did not phosphorylate the JNK and p38 substrates GST-Jun and GST-ATF2, respectively. Therefore, all subsequent assays were performed using the substrate histone H1. Interestingly, when compared to full length GST-SMAK, immunoprecipitated Myc-SMAK from transfected 293 cells displayed markedly higher autophosphorylation activity (Figure 6A). Treatment of active GST-SMAK (Figure 6B) or immunoprecipitated Myc-SMAK (not shown) with calf intestinal phosphatase (CIAP) caused a marked decrease in GST-SMAK activity, suggesting that its activity is regulated by phosphorylation. Furthermore these results indicate that SMAK is active in a hyperphosphorylated state.

Various growth factors, stress-inducing agents and apoptotic triggers have been demonstrated to affect the activity of several MAP kinases such as ERK1/2 as well as stress-activated kinases (Fanger et al., 1997; Davis, R.J., 1993; Xia, Z. et al., 1995; Hughes, D.A., 1995). To test whether SMAK activity could be modulated by such factors, a Myc epitope-tagged SMAK was transfected into 293 cells followed by stimulation with various factors and stress inducing agents in the absence of serum (see Figure 7A). The activity of SMAK following stimulation was evaluated by in vitro kinase assays. Interestingly, none of the factors tested were found to significantly modulate SMAK kinase activity in overexpressing 293 cells (Figure 7A), suggesting that SMAK is a component of a novel signaling pathway.

To gain insights into potential downstream effectors of SMAK, the relative kinase activity of JNK1 isoforms or the mitogen-activated protein kinases ERK1/2 was determined by immunoprecipitations and in vitro kinase assays following transient transfections of Myc-SMAK. As shown in figure 6C, JNK1 activity was found to be upregulated 3- to 5-fold relative to vector-transfected cells, suggesting that c-jun amino-terminal kinase1 (JNK1) is activated by SMAK overexpression. However, JNK activation by SMAK overexpression was about 3-fold less than that observed following UV irradiation (Figure 7C, lane 2). In contrast to JNK1, immunoprecipitated ERK1/2 were found to be inactive following SMAK transfection, suggesting that components of the mitogen response pathways are not markedly affected by SMAK overexpression (Figure 7C).

Materials and Methods For Immunoprecipitations and in vitro kinase assays of Examples 3

and 4

For growth factor and stress agent stimulation, transfected 293 cells were serum starved in DMEM for 1 hour prior to exposure to the stimuli. The cells were then exposed to the agonists as indicated in the figure legends and lysed as described above. For SMAK expression analysis, 20 mg of total cell lysate was subjected to western blotting with anti-SMAK antibodies as described above. For in vitro kinase assays, 100 mg of total cell lysate was immunoprecipitated with 1mg of 9E10 monoclonal antibodies and 20ml of G-protein sepharose (Pharmacia) for 2 hours at 4°C. Immunoprecipitates were washed three times with NETN (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) and once with kinase assay buffer (20 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 10 mM NaF, 10 mM b-glycerophosphate, 1 mM sodium orthovanadate). Reactions (20 ml) were initiated by the addition of 5 mCi of [g32P] ATP and 3 mg of histone H1. After a 30 minute incubation at 30°C, reactions were terminated by the addition of 4X SDS sample buffer and 20 ml aliquots were fractionated by 12% SDS-PAGE. Gels were stained, dried and exposed to X-ray films.

Recombinant GST-SMAK fusion protein was purified using glutathione sepharose as described (Pharmacia) from 10 ml cultures. GST-SMAK immobilized on beads was assayed directly for kinase activity on various substrates or treated with CIAP prior to kinase assays.

DISCUSSION OF EXAMPLES 1-4

The present inventors have cloned and characterized a murine Ste20-related protein kinase designated SMAK. Database searches revealed that the cloned cDNA encodes a protein kinase highly related to LOK, a Ste20-related protein kinase preferentially expressed in lymphocytes (Kuramochi, S. et al., 1997) . Because it was found to be related to Ste20 and shared extensive homology to microtubule and nuclear associated protein (M-NAP) and to AT1-46, we have termed this kinase SMAK. Analysis of the kinase domain shows that SMAK is also related to MST1/2 (Schinkmann, K and Blenis, J., 1997; Katoh, M. et al., 1995; Creasy, C.L. and Chernoff, J., 1995) and more distantly related to Ste20, a yeast kinase involved in the pheromone response pathway (Zhao, Z.S. et al., 1995). Furthermore, SMAK showed 70% identity to M-NAP and 63% and 71% identity to AT1-46 in two distinct carboxy terminal domains. Interestingly, LOK also display extensive homology to AT1-46 in its C-terminal domain, suggesting that AT1-46, SMAK and LOK represent members of a new protein family. Furthermore, the AT1-46 homology (ATH) domain may represent a novel protein motif required for SMAK and LOK function. Further analysis of SMAK protein showed the presence of an SH3-binding motif and a coiled-coil structure in the C-terminal region. Northern blot analysis demonstrated that SMAK is ubiquitously expressed in adult tissues and its expression appears to be restricted to more differentiated cell types.

Purified GST-SMAK fusion protein showed that it autophosphorylates efficiently and that it can phosphorylate exogenous substrates. However, the JNK and p38 substrates c-Jun and ATF2, respectively, were not phosphorylated by SMAK. Interestingly, alkaline phosphatase treatment of recombinant SMAK lead to a substantial decrease in kinase activity in vitro, suggesting that its activity is regulated by phosphorylation. Immunoprecipitations and in vitro kinase assays following overexpression of SMAK in 293 cells showed that it activated isoforms of JNK1. As for SMAK, the Ste20-related kinase p65PAK also activates the stress response pathway (Fanger, G.R. et al., 1997) . Similarly, activation of the related kinases MST1/2 activates the p38 MAPK pathway (Creasy, C.L. and Chernoff J., 1995; Graves, J.D. et al., 1998). However, treatment of transfected 293 cells with various growth factors and stress-inducing agents did not result in any significant changes in SMAK activity, suggesting that it is part of a novel pathway. One possibility is that SMAK possesses low kinase activity and is activated by autophosphorylation in vitro, making it difficult to observe any significant effects. Alternatively, SMAK is constitutively active and is downregulated through a novel signaling pathway.

Immunolocalization of Myc epitope-tagged SMAK protein revealed that it is localized predominantly to the periphery of C2C12 myoblasts. Interestingly, SMAK was found to be localized to distinct cytosolic domains. Whether these represent larger signaling complexes involving SMAK remains to be elucidated. One possibility is that the putative SH3-binding domain of SMAK mediates interactions with other proteins of such complexes.

__Example 5__

SMAK Overexpression Results in Rapid Induction of Apoptosis

Inspection of SMAK transfected cultures after 24 h revealed the presence of numerous cells that exhibited extensive cellular shrinkage, membrane blebbing, and loss of substrate adhesion. (Figure 9A). Moreover, several 9E10-positive cellular fragments, reminiscent of apoptotic bodies were evident following SMAK transfection into C2C12 cells (Figure 9A). Similar results were observed following transfection of SMAK into COS-1, HeLa, NIH3T3, and 293 cells (not shown). Taken together, these data suggested that forced expression of SMAK induced an apoptotic response.

To determine whether SMAK-transfected cells were exhibiting a bona fide apoptotic response, double staining was performed using 9E10 and FITC-labeled annexin V or terminal transferase dUTP nick end labeling (TUNEL) for the detection of early and late apoptotic stages, respectively. Double staining experiments performed 24 hours following Myc-SMAK transfection revealed the colocalization of FITC-annexin V, marking early apoptotic cells, and Myc-tagged SMAK protein (Figures 9C and 9D). Furthermore, over 75% of TUNEL-positive cells also expressed the transfected Myc-SMAK protein (Figures 9G and 9H). However, 48 hours after transfection, virtually no SMAK-expressing cells were

detectable. Taken together, these results demonstrate that forced expression of SMAK in C2C12 myoblasts triggered an apoptotic response. In addition, overexpression of the catalytically inactive mutant SMAKK63R also induced an apoptotic response (Figures 9E and 9F) raising the possibility that regions outside the kinase domain were of functional significance.

To gain insights into potential downstream effectors of SMAK, plasmids expressing c-Jun, Elk1 and CREB Gal4 fusions (Pathdetect plasmid system; Stratagene) were co-transfected with SMAK expression vectors together with a Gal4-Luciferase reporter gene. Transfection of SMAK with Gal4-Jun resulted in a 5 to 7-fold increase in luciferase activity relative to Gal4-Jun alone (Figure 10). This observation suggested that SMAK overexpression activated the c-jun amino-terminal kinase (JNK) pathway. However, JNK activation by SMAK overexpression was about 3-fold less than that observed with MEK kinase (MEKK), an upstream regulator of JNK . Relative to either effector transfected alone, a modest increase (2 to 3-fold) in reporter gene activity was observed when SMAK was co-transfected with Gal4-Elk or Gal4-CREB (Figure 4). Therefore, SMAK overexpression appeared to predominantly activate the JNK pathway. Supporting this, in vitro kinase assays revealed that active JNK1 was readily immunoprecipitated from SMAK-transfected 293 cells whereas ERK1/2 remained inactive (not shown).

Example 6

SMAK Overexpression Induces Actin Stress Fiber Dissolution

Focal adhesions are large protein complexes particularly prominent in cultured cells involved in cellular adherence to the substratum (Burridge and Chrzanowska-Wodnicka, 1996; Craig and Johnson, 1996; Jockusch et al., 1995; Schaller and Parsons, 1994). Focal adhesion complexes are enriched for proteins such as paxillin, vinculin and focal adhesion kinase (FAK) which modulate focal contact dynamics. In addition, the assembly of focal adhesions is regulated by the Rho family of small GTPases, which recruit and modulate the activity of several protein kinases through direct interaction (Burridge and Chrzanowska-Wodnicka, 1996; Nobes and Hall, 1995). The relatedness to Ste20 kinases such as p65PAK and the cellular distribution of SMAK to distinct cytosolic foci predominantly along the periphery of the cells raised the possibility that SMAK may modulate focal adhesion dynamics. To investigate this possibility, actin stress fibers in SMAK-transfected C2C12 cells were detected by phalloidin staining. For comparison, cells were transfected with activated RhoA, which promotes stress fiber and focal complex assembly (Nobes and Hall, 1995), as well as activated Rac1 and p65PAK, both previously shown to induce actin stress fiber disassembly (Nobes and Hall, 1995; Van Aelst and D'Souza-Schorey, 1997).

Transfected myoblasts overexpressing SMAK showed almost a complete absence of stress fibers together with redistribution of actin to the cell periphery (Figures

11A and 11B). This observation suggested that SMAK induced stress fiber dissolution and actin reorganization. The catalytically inactive SMAK mutant SMAKK63R also promoted stress fiber dissolution, suggesting that SMAK-mediated actin reorganization was independent of kinase activity (not shown). The disassembly of stress fibers induced by SMAK was comparable to that observed for the activated form of Rac1, Rac1G12V (Figures 11C and 11D). Similarly PAKDE, an activated form of p65PAK (Manser et al., 1997), also mediated the dissolution of stress fibers in C2C12 myoblasts (Figures 10E and 10F). Overexpression of the stress fiber inducer RhoAG14V in C2C12 cells similarly induced apoptosis (see Figures 12E and 12F). Taken together, these data raise the possibility that Rho GTPases and p65PAK represent potential regulators or effectors of SMAK.

The observation that Rho GTPases, p65PAK, and SMAK promoted stress fiber reorganization in C2C12 myoblasts prompted us to examine whether they localized to similar sites within the cell. Myoblasts were transfected with Myc epitope-tagged versions of both SMAK and p65PAK and immunolocalized using 9E10 antibodies. The small GTPases RhoA and Rac1 were similarly transfected. Interestingly, PAK and SMAK displayed a very similar pattern of staining with reactivity at the periphery of transfected myoblasts (Figures 12A and 12B). Similarly, transfection of the dominant negative RhoAT19N also resulted in distinct domains of reactivity predominantly at the periphery of the cells (Figure 12C). In contrast, Rac1 displayed a uniform cytosolic distribution (Figure 12D). In contrast to HeLa cells, overexpression of the activated RhoAG14V resulted in morphological changes characteristic of apoptosis in myoblasts (Figures 12E and 12F). Interestingly, overexpression of p65PAK in C2C12 myoblasts also resulted in characteristic apoptotic morphology (see arrow Figure 12B). Whether this is due to cellular retraction following stress fiber dissolution or activation of PAK through caspase proteolytic cleavage is unclear.

Recently, aPAK (hPAK1) was shown to colocalize with Rac1 and Cdc42 to peripheral focal complexes in transfected HeLa cells. In addition, overexpression of aPAK in HeLa cells induces the loss of actin stress fibers (Manser et al., 1997). In C2C12 myoblasts, the cellular distribution of Myc epitope-tagged versions of p65PAK, RhoAT19N and SMAK are strikingly similar (Figures 12A-F). Therefore, these observations raise the possibility that PAK, RhoA and SMAK are part of the same complex. To test this, we constructed a HA-tagged SMAK expression vector and performed cotransfections experiments with Myc-tagged PAK or RhoAT19N plasmid vectors. Transient transfections and double labeling experiments clearly indicate that PAK and RhoA colocalized with SMAK at peripheral as well as internal sites (Figures 12G-L). Therefore, our data are consistent with the hypothesis that PAK, RhoA, and SMAK are components of a peripheral focal adhesion complex.

Example 7

Induction of Apoptosis and Actin Disassembly are Separable Activities

The observation that the kinase inactive SMAKK63R promoted actin reorganization suggested that stress fiber dissolution by SMAK was independent of kinase activity. To investigate this hypothesis, we constructed vectors expressing Myc-tagged wildtype and mutant SMAK truncations and performed phalloidin staining of transfected C2C12 cells. Two of the vectors, SMAKΔC and SMAKK63RΔC contained the wildtype and mutant SMAK kinase domain from residues 1-372, extending slightly into the M-NAP region, 66 residues short of the caspase cleavage site (see Figure 13 for plasmid vectors). Strikingly, this truncation displayed about a 10-increase in activity compared to full length SMAK in an in vitro kinase assay using both immunoprecipitated Myc-SMAK kinase (see Figure 14J) and recombinant GST-SMAK fusion proteins purified from bacteria (not shown). Therefore, these data strongly suggest that the carboxy-terminal region contains a negative autoregulatory domain that normally inhibits kinase activity.

Relative to wildtype SMAK, overexpression of SMAKΔC in C2C12 cells resulted in a markedly increased rate of apoptosis as evidenced by a large increase in numbers of cells exhibiting cellular shrinkage and membrane blebbing 16 hours following transfection (Figures 14A and 14B). By contrast forced expression of the kinase-inactive SMAKK63RΔC did not induce cell death, suggesting that the enhanced apoptotic response was due to activation of the kinase domain (Figure 14C). However, overexpression of SMAKK63RΔC did not result in any apparent loss of stress fibers following transfection (Figure 14D). Consistent with the observation that SMAK-mediated actin reorganization was independent of kinase activity, a mutant lacking the kinase domain up to residue 372 (termed SMAKΔN, see Figure 13), strongly promoted stress fiber disassembly (Figures 14E and 14F). In addition, SMAKΔN-transfected cultures contained a high proportion of retracting cells, suggesting that the SMAK C-terminal domain relative to full length SMAK is a potent effector of stress fiber dissolution.

To evaluate the rate of apoptosis induced by the various Myc-tagged SMAK vectors, the proportion of annexin V and 9E10 double positive cells was measured relative to the total number of 9E10-positive cells. As shown in Figure 14I, cultures transfected with the active SMAKΔC displayed about a 6-fold increase in numbers of double-positive apoptotic cells 16 hours post-transfection compared to cultures transfected with SMAKΔCK63R or control vector. Transfection with SMAKΔN resulted in a frequency of double positive apoptotic cells that was about 3-fold less than that observed in cultures transfected with SMAKΔC and similar to that observed in cultures transfected with full length SMAK (not shown). Transfection with SMAKΔC resulted in a 3-fold higher rate of induction of apoptosis relative to cells transfected with SMAKΔN (Figure 14G). In summary, these results suggest that both N- and C-terminal domains of SMAK were capable of inducing apoptosis. Forced expression of the N-terminal kinase domain resulted

in a rapid and efficient induction of apoptosis that was dependent on kinase activity. By contrast, forced expression of the C-terminal actin disassembling region resulted in stress-fiber dissolution followed by a delayed induction of apoptosis presumably due to cellular retraction and loss of adhesion.

Example 8

The ATH Domain Mediates Actin Disassembly

To further delineate the domains that mediate stress fiber disassembly, a series of Myc-SMAK deletions and truncations were generated and evaluated for their ability to reorganize actin stress fibers (Figure 13). SMAKΔ3′ bears a carboxy-terminal deletion of 263 amino acids, removing part of the ATH domain. Construct pXX1.2 encompasses SMAK amino acids 551-950 spanning part of the kinase domain extending into the M-NAP region. Construct pBg2631 contains amino acids 856-1202, extending to the end of the ATH domain. Finally, pXh2973 encodes the last C-terminal 263 amino acids of the ATH region that were deleted in SMAKΔ3′. Expression plasmids were transiently transfected into C2C12 cells and the cultures fixed and processed for 9E10 and phalloidin staining.

Consistent with the notion that the carboxyl 263 amino acids negatively regulates actin polymerization, overexpression of SMAKΔ3′ led to a markedly increased density of actin stress fibers (Table 1). Transfection of SMAKΔ3′ nevertheless resulted in an apoptotic response marked by shrinkage and membrane blebbing (not shown). Transfection of pXX1.2 resulted in a nuclear associated staining pattern together with an increased density of strongly staining stress fibers, but not in an induction of apoptosis (Table 1, data not shown). Therefore, we conclude that the ATH region of SMAK is required to effect actin reorganization and the subsequent induction of apoptosis. Transfection of pBg2631, bearing a partial deletion of the M-NAP region, but encompassing the ATH domain, efficiently effected the disassembly of actin stress fibers and thereafter induced apoptosis (Table 1).

Taken together, transfection of deletion constructs mapped the stress fiber disassembling domain of SMAK to the last carboxy-terminal 263 amino acids. To confirm this, pXh2973 was constructed and transfected into C2C12 cells. Phalloidin staining of pXh2973-expressing cells revealed a dramatic loss of actin fibers (Figures 13G and 13H and Table 1). In addition, most cells expressing Xh2973 displayed a morphology reminiscent of cellular retraction, indicating that SMAK-mediated disassembly of actin fibers lead to a loss of substrate adherence. Furthermore, the presence of 9E10-positive apoptotic cells observed in Xh2973-transfected cultures supports the contention that the loss of actin stress fibers irrevocably leads to cell death. Therefore, we conclude that the ATH domain is necessary and sufficient to induce stress fiber dissolution.

Example 9

Caspase-3 Cleavage Stimulates Kinase Activation

Inspection of the SMAK protein sequence revealed the presence of a caspase-3 consensus cleavage site D-T-Q-D436 at amino acid position 436. This observation together with the finding that SMAK mediated apoptosis and stress fiber disassembly through distinct domains, raised the possibility that SMAK represents a novel caspase-3 substrate.

To investigate this possibility, full length wildtype SMAK and the caspase-3 cleavage site mutant SMAKD436N were translated in vitro in the presence of 35S-methionine. The translation products were incubated with crude lysates from bacteria expressing recombinant caspase-3, or alternatively, with lysates from Rat1-Myc/ER cells triggered to undergo apoptosis , then analyzed by SDS-PAGE. As shown in Figure 15A, in vitro translated SMAK displayed a complex banding pattern due to the presence of incomplete translation products. Nevertheless, caspase-3 cleavage products of approximately 133 and 60 kDa were observed following incubation of SMAK with recombinant caspase-3 (Figure 15A; lanes 3 and 7). Mutation of the cleavage site at residue 436 resulted in an inability of caspase-3 digestion to liberate the 133 and 60 kDa fragments (Figure 15A; lanes 4 and 8). Addition of the caspase-3 inhibitor Z-DEVD-fmk, completely inhibited the release of both fragments from wildtype SMAK indicating that cleavage is mediated by a caspase-3-like activity (Figure 15A; lanes 5). Lastly, incubation of wildtype SMAK or SMAKD436N with an apoptotic cell lysate resulted in a similar banding pattern, which was abrogated by the addition of Z-DEVD-fmk (Figure 15A; lanes 6-9). Therefore, we conclude that caspase-3 specifically cleaves SMAK in vitro at residue 436.

The cleavage of SMAK by a caspase-3-like activity following incubation with an apoptotic cell lysate raised the possibility that SMAK is an in vivo substrate for caspase-3 during an apoptotic response. To address this, Rat1-Myc/ER cells expressing a Myc-estrogen receptor fusion were induced to undergo apoptosis by the addition of -estradiol and extracts subjected to Western blot analysis using different anti-SMAK polyclonal antibodies. Western analysis with anti-SMAK1 antibody, directed against the kinase domain and the M-NAP region, revealed that the 133 and 60 kDa SMAK cleavage products increased over time following the onset of Myc-induced apoptosis (Figure 15B). Analysis of the same extracts with anti-SMAK2, a kinase domain-specific antibody, resulted in the detection of the 60 kDa cleavage fragment 12 hours following the addition of -estradiol (Figure 15C; lanes 4 and 5). Anti-SMAK2 antibodies did not detect the 133 kDa fragment, suggesting that it represents the C-terminal domains of SMAK (not shown). Western blot analysis of caspase-3-treated N-terminal Myc-tagged SMAK with antibody 9E10 also detected a 60 kDa product (data not shown). Similarly, release of the 60 kDa product from the endogenous SMAK protein was observed when NIH3T3 cells were exposed to apoptotic triggers such as TNF- and UV irradiation (Figure 15C; lanes 6-9). Therefore, we conclude that caspase-3 cleavage of SMAK in vivo releases the kinase domain as an N-terminal 60 kDa product and this cleavage represent a common step in response to various

apoptotic stimuli. As shown previously, SMAKΔC was found to display higher activity than wild type SMAK in an in vitro kinase assay. Surprisingly, the cleavage site mutant SMAKD436N showed no kinase activity in vitro, suggesting that the caspase-3 cleavage site is required for SMAK activity. One possible explanation is that overexpression of SMAK in cells induces actin fiber disassembly which triggers an apoptotic response resulting in caspase activation and subsequent SMAK cleavage in a feedback loop. Therefore, the D436N mutation results in a non-cleavable inactive kinase.

To investigate the effect of caspase-3 cleavage on SMAK kinase activity, various SMAK mutants were transfected into 293 cells, immunoprecipitated and assayed for kinase activity following treatment with recombinant caspase-3 in vitro. As shown in Figure 15D, exposure of wildtype or truncated SMAK proteins to recombinant caspase-3 resulted in a marked increase in kinase activity, suggesting that caspase-mediated cleavage of SMAK activated protein kinase activity. By contrast, only a slight increase in kinase activity was observed following incubation of SMAKD436N with caspase-3. This small increase is likely due to limited non-caspase proteolytic degradation, as breakdown products were observed following incubation of in vitro translated SMAKD436N with the caspase-3 inhibitor Z-DEVD-fmk (see Figure 15A). Shown in Figure 15E is a Western blot demonstrating expression of the different SMAK mutants used in the kinase assays (Figure 15D) following transfection into 293 cells.

<u>Materials and Methods For Examples 5-9</u>

Cell Culture, Transfection, and Luciferase Assays

C2C12 cells were maintained in Dulbecco's modified Eagle medium supplemented with 15% fetal calf serum (FCS). Rat1-Myc/ER, NIH3T3, and 293 cells were grown in DMEM containing 10% FCS. For luciferase assays and stimulation of 293, the cells were plated at 1 x 105 / 35 mm dish 24 hours prior to transfection. The cultures were transfected by Lipofectamine (Gibco/BRL) according to the manufacturer's instructions using 1 μg of Gal4-Luciferase reporter, 1 μg of SMAK expression vector and 100 ng of effector plasmid. For luciferase assays, 293 cells were harvested 18-20 hours following transfection and the cells were lysed using reporter lysis buffer (Gibco/BRL). Equivalent portions of extracts were assayed on a Lumat-100 luminometer using 100 μl of Luciferase assay reagents (Promega). The average of five independent experiments, performed in duplicate and normalized to protein concentration, is shown.

Northern Analysis, Immunofluorescence, and Apoptosis Assays

Analysis of SMAK protein expression was performed by lysing the cultures (150 mM NaCl, 50 mM Tris, pH 7.4, 1 mM EDTA, 1% Triton-X 100 and 1 μg/ml of each aprotinin, pepstatin and leupeptin) and subjecting 20 μg of total lysate to western blot analysis using anti-SMAK rabbit polyclonal antibodies . Reactive proteins were detected by ECL (Amersham) using a goat anti-rabbit horse radish peroxidase (HRP) labeled

secondary antibody. SMAK polyclonal antibodies were generated by immunization of New Zealand rabbits using purified GST-SMAK95-551 (anti-SMAK1) encompassing part of the kinase and M-NAP domains, or GST-SMAK1-93 (anti-SMAK2; kinase domain). Specific immunoreactivity to murine SMAK protein was observed in 293 cells transfected with full length and truncated SMAK expression vectors.

For expression studies and immunolocalization experiments, HA- or Myc-tagged pcDNA3 (Invitrogen) expression vectors bearing full length or truncated SMAK were constructed using standard cloning procedures . Briefly, SMAKΔC was constructed by deleting amino acids 373-1202, leaving the kinase domain. The SMAKΔN deletion was generated by deletion of the first 372 amino acids of SMAK. The kinase dead version, SMAKK63R, and the cleavage mutant SMAKD436N, were obtained through PCR-based mutagenesis of the ATP binding site at residue 63 and the aspartic acide at position 436, respectively. Plasmid SMAKΔ3′ was created by deleting the last c-terminal 263 amino acids of Myc-SMAK. Plasmid pXX1.2 encompasses amino acids 551-950. The expression vector pBg2631 contains amino acids 856-1202. Finally pXh2973 was generated by inserting a fragment encompassing the last 263 amino acids of SMAK into Myc-tagged pcDNA3.

Following transfection into C2C12, the cultures were fixed for 10 minutes in 4% paraformaldehyde and SMAK protein was detected using 9E10 or rabbit anti-HA (Santa Cruz) antibodies in conjunction with FITC- or TRITC-labeled secondary antibodies. Actin stress fibers were detected using 220 nM TRITC-phalloidin (Sigma) on fixed cultures for 15 minutes. Cells undergoing apoptosis were detected using annexin V-FITC (Oncor) or by TUNEL staining (Oncor) according to the manufacturer's specifications.

Caspase-3-expressing bacterial lysates or Rat1-Myc/ER lysates were prepared according to Song et al. (1997). Cleavage assays on immunoprecipitated or in vitro labeled (Promega TNT System) SMAK proteins were performed as described (Song et al., 1997).

Immunoprecipitations and In Vitro Kinase Assays

Bacterially expressed GST-SMAK and immunoprecipitated Myc-SMAK autophosphorylated and efficiently phosphorylated MBP and histone H1 in vitro (not shown). However, SMAK did not phosphorylate the JNK and p38 substrates GST-Jun and GST-ATF2, respectively. Therefore, all assays were performed using histone H1 or MBP. For in vitro kinase assays, of total cell lysate were immunoprecipitated with 1 μg of 9E10 monoclonal antibodies and 20 μl of G-protein sepharose (Pharmacia) for 2 h at 4oC. Immunoprecipitates were washed three times with NETN (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40) and once with kinase assay buffer (20 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 10 mM NaF, 10 mM b-glycerophosphate, 1 mM sodium orthovanadate). Reactions (20 μl) were initiated by the addition of 5 μCi of [g32P] ATP and 3 μg of histone H1. After a 30 min incubation at 30°C, reactions were terminated by the

addition of 4X SDS sample buffer and 20 μl aliquots were fractionated by 12% SDS-PAGE. The gels were stained, dried and exposed to X-ray films. For coupled caspase-3 cleavage/kinase assays, immunoprecipitated SMAK proteins were incubated at 37°C in the presence or absence of caspase-3 lysates and then washed extensively with NETN and subjected to kinase assays using histone H1 as above.

__Discussion of Examples 5-9__

SMAK Induces Apoptosis and Stress Fiber Disassembly

Immunolocalization of Myc epitope-tagged SMAK protein revealed that it was localized predominantly to the periphery of C2C12 myoblasts. These regions are highly enriched in focal adhesion proteins such as FAK and vinculin (Craig and Johnson, 1996; Jockusch et al., 1995). Transfected cells exhibited cellular shrinkage and membrane blebbing suggestive of programmed cell death. Staining of the transfected cultures for specific apoptotic markers such as membrane inversion and DNA fragmentation indicated that SMAK rapidly induced an apoptotic response. Similarly, the recently identified but unrelated ZIP, RIP, and ASK1 kinases also induce cell death (Ichijo et al., 1997; Kawai et al., 1998; Stanger et al., 1995). However, an inactive form of SMAK was still able to elicit an apopotic response, suggesting that SMAK induced programmed cell death in a kinase-independent manner. This notion was confirmed by the observation that the C-terminal ATH region (lacking the kinase domain), was sufficient to induce an apoptotic response, but with delayed kinetics.

Interestingly, apoptotic membrane blebbing is regulated in part by myosin light chain (MLC) phosphorylation and inhibitors of MLC kinase decrease membrane blebbing (Mills et al., 1998). In addition, MLC kinase activity is regulated by the Rho-binding kinase ROK suggesting a role for p21-activated kinases in the process of cytoskeletal reorganization during apoptosis. Supporting this is the observation that overexpression of p65PAK mutants in Fas-triggered Jurkat cells induces cell death but without the formation of apoptotic bodies (Rudel and Bokoch, 1997). Extensive membrane blebbing was induced following transfection of the SMAK kinase domain and this effect was kinase-dependent. Therefore, an interesting possibility is that SMAK has a unique role in the regulation of cellular remodeling during programmed cell death.

Focal adhesions are a dynamic protein complex involved in cellular adherence to the substratum (Craig and Johnson, 1996). The turnover of focal complexes and actin stress fibers is coupled to the activity of the Rho family of small GTPases (Burridge and Chrzanowska-Wodnicka, 1996; Ilic et al., 1997); (Narumiya et al., 1997). Several GTP-Rho binding proteins have been identified including p140mDia and the protein kinases p160ROCK, ROK a, MRCK a, PKN, and PRK2. These GTP-Rho binding proteins appear to regulate cytoskeletal reorganization by promoting stress fiber formation. By contrast, overexpression of a-PAK induces loss of both focal adhesions and actin stress fibers (Manser

et al., 1997). Overexpression of the tumor suppressor and protein phosphatase PTEN also downregulates the formation of focal adhesions (Tamura et al., 1998). Although SMAK does not contain any homology to known GTPase binding domains, overexpression of both SMAK and an kinase-inactive mutant resulted in actin stress fiber dissolution, suggesting that this is a kinase-independent process (Kawai et al., 1998). Moreover, an N-terminal deletion of SMAK lacking the kinase domain efficiently promoted stress fiber disassembly and induced characteristic apoptotic morphology in transfected C2C12 cells. Similarly, exposure of C2C12 myoblasts to 2-chloro adenosine induces disruption of actin microfilaments and triggers apoptosis (Rufini et al., 1997). One possibility is that SMAK is titrating important factors required for the maintenance of focal adhesions or actin stress fibers. Functional deletion analysis of SMAK revealed that overexpression of the C-terminal ATH domain of SMAK led to stress fiber dissolution and cellular retraction, suggesting that this domain negatively regulates stress fiber formation. The ATH region may be interfering with regulatory components of focal complexes, supporting a role for SMAK in the regulation of stress fiber dynamics.

The Ste20-related kinase, PAK, has been shown to be recruited to focal adhesion sites by activated Cdc42 and Rac1 (Manser et al., 1997). Interestingly, SMAK colocalizes with PAK and RhoA in transfected C2C12 myoblasts, suggesting that they are part of the same protein complex. The presence of a putative SH3-binding domain in the M-NAP region of SMAK represents an attractive target for docking onto such a protein scaffold. The Ste20-related protein kinase HPK1 has been demonstrated to bind directly to the SH2/SH3 containing adapter protein Grb2 and to be recruited to the autophosphorylated EGF receptor (Anafi et al, 1997), providing a mechanism for cross-talk between distinct biochemical pathways. Whether activated p21s impinge on SMAK activity is currently being investigated. However, preliminary results suggest that SMAK and Rac/PAK are components of independent pathways (L. A. Sabourin and M.A. Rudnicki, unpublished observations).

The Rho-associated kinase ROK, has been shown to phosphorylate proteins of the ezrin/radixin/moesin family (ERM), localized within focal complexes and to regulate their association (Matsui et al., 1998). In addition ROK is involved in the control of MLC kinase activity and membrane blebbing through direct regulation of myosin phosphatase (Kimura et al., 1996). Recently, LIM-kinase has been implicated in the control of cytoskeleton reorganization through phosphorylation of cofilin, a ubiquitous actin binding protein required for actin depolimerization (Arber et al., 1998; Yang et al., 1998). Therefore, to understand its role during actin reorganization, it will be of interest to identify SMAK substrates. In addition, the identification of targets for the ATH domains will provide valuable clues as to the mechanisms by which SMAK regulates stress fiber dynamics.

Caspase Cleavage of SMAK Releases Distinct Functional Domains

Although there are several kinases that mediate cell growth, only a few have been identified that trigger apoptosis. Recently, JNKs have been shown to be activated by apoptotic triggers such as Fas ligand and TNF-a. ASK1, a MAP kinase kinase kinase, has been shown to induce cell death and to activate JNK and p38 MAP kinase (Ichijo et al., 1997). Recently, the SMAK related kinases MST1 and PAK2 have been shown to be substrates for caspase-3. Caspase-mediated cleavage was demonstrated to activate their kinase activity. In addition, MST1 has been shown to activate MKK6, MKK7, p38 and stress activated protein kinases (Graves et al., 1998). However the mechanisms by which MST1 activates these kinases are unknown.

Similar to p65PAK and MST1 (Graves et al., 1998; Lee et al., 1998; Lee et al., 1997; Rudel and Bokoch, 1997), SMAK is a substrate for caspase-3 and is rapidly cleaved following the induction of apoptosis. Furthermore, cleavage releases an activated SMAK kinase domain and an actin fiber disassembling region that appear to function independently.

Several studies have demonstrated that actin fiber disassembly and cytoskeletal rearrangements represent significant steps in the process of apoptosis (Brancolini et al., 1997; DeMeester et al., 1998; Ghosh et al., 1997; Kletsas et al., 1998; Mills et al., 1998; Palladini et al., 1996; Rufini et al., 1997). Furthermore, actin has been demonstrated to be resistant to caspase cleavage during apoptosis, suggesting that it is required for cellular remodeling during the apoptotic process and that reorganization mechanisms need to be activated (Rice et al., 1998; Song et al., 1997; Villa et al., 1998). Therefore, SMAK may represent a novel pro-apoptotic effector for which caspase cleavage releases a cytoskeletal disassembling function concomitant with kinase activation. Whether the active kinase domain is involved in cytoskeletal remodeling, apoptotic signaling or both, remains to be determined. Interestingly, disruption of the actin filament network by cytochalasin D activates p53-dependent transcription and apoptosis Conversely, activation of p53 through SV40 large T antigen inactivation also leads to F-actin disassembly (Guenal et al., 1997).

Actin stress fibers are ultimately anchored at focal adhesion sites through interactions with proteins such as a-actinin, vinculin and talin (Craig and Johnson, 1996). Interestingly, overexpression of gelsolin, an actin-regulatory protein found at focal sites has been demonstrated to protect Jurkat cells from Fas-induced apoptosis by preventing changes in the F-actin morphology and inhibition of caspase-3 (Ohtsu et al., 1997). However, endogenous gelsolin protein was found to be a substrate for caspase-3. Caspase-3-cleaved gelsolin was demonstrated to destabilize the actin network, causing cellular retraction, detachment and apoptosis (Kothakota et al., 1997). Similarly, the product of the growth arrest-specific 2 (Gas2) gene is also cleaved by an ICE-like protease activity during

apoptosis (Brancolini et al., 1995). Gas2 is known to be associated with the actin microfilament network and caspase cleavage induces its actin reorganization activity (Brancolini et al., 1995). One attractive possibility is that the caspase-3-mediated release of the ATH domain interferes directly with the function of actin-regulatory proteins such as gelsolin or Gas2 or alternatively, may promote their proteolytic processing.

The present inventors have cloned and characterized a Ste20-related kinase, SMAK, which can mediate apoptosis and promote stress fiber dissolution. Although the full length protein can mediate both effects, the individual N- and C- terminal domains were more efficient at inducing apoptosis and actin reorganization respectively. We have shown that SMAK is a substrate for a caspase-3-like activity in vivo during the process of apoptosis. Furthermore, caspase-3-mediated cleavage of SMAK increased its intrinsic kinase activity. These results raise the interesting possibility that kinases such as SMAK and PAK may represent a new class of dual function proteins playing important roles in the regulation of the apoptotic response as well as cytoskeleton reorganization. The identification of SMAK substrates, regulatory molecules and interacting partners will provide further insights into mechanisms underlying its regulation during the process of actin reorganization and programmed cell death.

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications referred to herein are incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Table 1

Expression Vector	Actin Disassembly	Kinase Activity	Apoptosis	Cellular Localization
SMAK	+	+	+	CP
SMAKΔC	-	+++	+++	DC
SMAKΔN	+	-	+	DC
SMAKΔ3'	-*	+	+	DC
XX1.2	-*	-	-	PN
Bg2631	+	-	+	DC
Xh2974	++	-	+	DC
$SMAK^{K63R}$	+	-	+	CP

Table 1. Actin dissembling and apoptosis-inducing activities of SMAK mutants and N- and C-terminal truncations. Following transfection into C2C12 cells, the cultures were fixed and stained using anti-Myc and TRITC-phalloidin to evaluate their actin disassembling activity. The relative kinase activities were determined by Immunoprecipitations and *in vitro* kinase assays following transfections in 293 cells.

Abbreviations: CP, cell periphery; DC, diffuse cytoplasmic; PN, perinuclear.

* An increased density of stress fibers was observed following transfection of SMAKΔ3' and XX1.2.

DETAILED FIGURE LEGENDS

Figure 1. Nucleotide and amino acid sequence of murine SMAK. The N-terminal kinase domain is highlighted in black. The M-NAP and AT1-46 regions are shaded and boxed, respectively. The putative SH3-binding motif has been highlighted in white within the M-NAP domain.

Figure 2. Deduced amino acid sequence of murine SMAK. The N-terminal kinase domain is highlighted in black. The M-NAP and AT1-46 regions are shaded and boxed, respectively. The putative SH3-binding motif has been highlighted in white within the M-NAP domain.

Figure 3. Sequence alignment of SMAK with related proteins. (A) Alignment of SMAK kinase domain with that of the Ste20-related kinases human SLK (hSLK), LOK and MST. The kinase subdomains are numbered I-XI and conserved residues within the kinase domain are marked by an asterisk. The characteristic Ste20 motif in subdomain VIII is underlined. (B) Alignment of SMAK with Rat SLK, LOK, M-NAP and AT1-46. In both alignments, the shaded area represent identical amino acid residues. Numbers on the left indicate the amino acid residues. (C) Schematic representation of SMAK and similarity indices (%) to various other polypeptides. The catalytic domain (black) is most similar to LOK and MST1/2, two Ste20-like kinases. SMAK is highly homologous to microtubule and nuclear associated protein (M-NAP; grey) and to AT1-46 (white) in the carboxy terminal region. Numbers in brackets represent SMAK1 amino acid residues.

Figure 4. Northern blot analysis of tissues and cell lines. 4 µg of poly-A+ RNA from various tissues was analyzed for SMAK expression and normalized for b-actin mRNA levels. Northern analysis shows the presence of at least 3 SMAK mRNA species of approximately 6,7 and 8 kb in length. The 7kb isoform being predominantly expressed in all the human tissues surveyed. (B) Expression analysis of murine tissue RNA. 4 µg of poly-A+ RNA from various human tissues (Clonetech MTN) was analyzed for SMAK mRNA levels and normalized to b-actin expression. Northern analysis shows the presence of at least 3 SMAK mRNA species of approximately 6, 7 and 8 kb in length. The largest isoform being predominately expressed in all the murine tissues surveyed. (C) SMAK mRNA expression in murine cell lines. 4 µg of poly-A+ RNA from four murine cell lines were surveyed for SMAK mRNA levels. In contrast to adult tissues, cultured cells expressed similar amounts of all three SMAK mRNA isoforms. Interestingly, the embryocarcinoma cell line P19 showed no detectable levels of SMAk1 mRNA. (D) Analysis of SMAK expression in various human cell lines. 2 µg of poly-A+ RNA from different human tumor cell lines (Clonetech MTN) were analyzed for SMAK expression. Relative to normal blood lymphocytes and colon tissue,

respectively, high levels of SMAk1 mRNA were observed in K562 cells, a chronic myelogenous leukemia cell line and SW480, a colon adenocarcinoma.

Figure 5. Expression of SMAK during C2C12 differentiation and serum stimulation of NIH3T3 fibroblasts. (A) C2C12 cells were induced to differentiate in DMEM containing 2% horse serum (DM) and harvested at 0, 1, 3 and 5 days following the induction of differentiation. 20 µg of total cell lysate was analyzed for SMAK expression by immunoblotting using an anti-SMAK rabbit polyclonal antibody. A significant decrease in SMAK protein levels is observed following the transfer of the cells to differentiation medium. 293/SMAK and 293/SMAKΔ3′ represent antibody control lysates from 293 cells transfected with a full length and a carboxy terminal truncated version of SMAK. (B) NIH 3T3 cells were serum starved for 24 hours and then stimulated with 10% fetal calf serum for 1 day and total cell lysates were analyzed for SMAK protein levels. A rapid decrease in SMAK levels was observed in serum starved cells. However, following serum stimulation, SMAK levels were up-regulated.

Figure 6. In Vitro kinase assay of purified or immunoprecipitated SMAK. (A) Bacterially expressed full length SMAK (lane A) or immunoprecipitated Myc-tagged SMAK (lane B) were assayed for kinase activity on various substrates. Both showed activity on MBP and histone H1. No detectable activity was observed on GST-Jun or GST-ATF2. SMAK protein purified from bacteria was approximately 10 times more active on these substrates relative to the kinase immunoprecipitated from transfected 293 cells. (B) Inactivation of SMAK by calf intestinal alkaline phosphatase (CIAP) treatment. Purified recombinant GST-SMAK protein was subjected to 2 and 20 units of CIAP for 30 minutes at 37°C, washed and assayed for activity. Decreased kinase activity was observed following CIAP treatment.

Figure 7. Effect of extracellular stimuli on SMAK activity. (A) Immunoprecipitation and in vitro kinase assay of transfected Myc-SMAK. Following transfection of 293 cells, the cultures were starved for 1 hour in serum free medium and then stimulated with various agents for 15 minutes. The cells were then lysed and assayed for SMAK activity on histone H1 in vitro. Lane 1: untransfected 293, lane 2: 10% FCS, lane 3: 300 nM TPA, lane 4: 100 ng/ml EGF, lane 5: non-starved, lane 6: 10 M A23187, lane 7: UV (200J/m2), lane 8: 100 ng/ml TNF-a, lane 9: 20 ng/ml IL1-a, lane 10: unstimulated. No change in SMAK activity was detected after exposure to the various stimuli. (B) Immunoblot analysis shows that the cultures expressed equivalent amounts of transfected SMAK protein. (C) Activation of JNK1 by SMAK. 293 cells were transiently transfected with Myc-SMAK and JNK1 or ERK1/2 was immunoprecipitated 18 hours later using an anti-human JNK1 (Pharmingen) or ERK1/2 (Santa Cruz) antibody. Kinase assay was performed on GST-Jun (JNK) or MBP (ERK1/2).

Figure 8. Immunolocalization of SMAK in C2C12 myoblasts. C2C12 cells were transiently transfected with a Myc-epitope tagged SMAK vector and stained at various times after transfection for expression using 9E10 supernatant. Distinct clusters of staining can be observed at the periphery of the cells 16 hours after transfection for both the wild type (A) and SMAKK63R, a mutant form of SMAK (B). Photomicrographs are shown at 400X.

Figure 9 shows induction of Apoptosis in SMAK Transfected C2C12 Myoblasts

(A) Detection of Myc-SMAK 24 h following transfection revealed that almost all 9E10 labeled cells exhibited membrane blebbing and cell shrinkage suggesting that SMAK induced cell death.

(B) Phase contrast photomicrograph of C.

(C and D) Double immunofluorescent staining of Myc-SMAK and annexin V-FITC in transfected C2C12 cells indicating that SMAK transfection had induced apoptosis. Arrows denote identical cells in E and F.

(E and F) Immunodetection of the Myc-SMAKK63R lacking functional kinase activity in transfected C2C12 cells and co-localization of annexin V-FITC indicated that kinase activity was not required for full length SMAK to induce apoptosis. Arrows denote identical cells in G and H.

(G and H) Immunodetection of Myc-SMAK expression in cells undergoing DNA fragmentation as detected by TUNEL labeling supports the contention that forced expression of SMAK induces apoptosis. Arrows denote identical cells in G and H.

Figure 10 shows SMAK Induces the Stress Activated Signaling Pathway

Gal4-Luciferase reporter and Gal4-Elk1, Gal4-Jun and Gal4-CREB fusions with or without SMAK were transiently transfected into 293 cells. Cotransfection of SMAK and Gal4-Jun resulted in a 5 to 7-fold increase in luciferase activity. By contrast, cotransfection of SMAK and Gal4-Elk and Gal4-CREB resulted in a 2-fold increase in luciferase activity. The data shown represent the average +/- SEM from 5 independent experiments using duplicate samples, normalized to protein concentrations.

Figure 11. Overexpression of SMAK Induces stress fiber dissolution

(A and B) Expression of Myc-SMAK in transiently transfected C2C12 cells induced loss of actin stress fibers as detected by phalloidin staining. Arrow denotes the identical cell in panels A and B.

(C and D) Expression of Myc-RacV12 in C2C12 cells similarly induced loss of stress fibers as evidenced by phalloidin staining. Arrow denotes the identical cell in panels C and D.

(E and F) Expression of transfected Myc-p65PAK also induced loss of actin stress fibers. Arrow

denotes the identical cell in panels E and F.
The cells were fixed and stained 16 hours post transfection with anti-Myc antibody 9E10 and TRITC-Phalloidin, and photographed at 400X.

Figure 12. Colocalization of SMAK, PAK, and RhoA in transfected C2C12 myoblasts
(A-D) Immunodetection of transfected Myc-tagged SMAK, PAK, RhoAN19 and Rac1V12, respectively with antibody 9E10. Note the similar pattern of punctate staining at the cell periphery.
(E) Induction of cell death by activated RhoA in C2C12 cells. RhoAV14 expression was detected using 9E10 16 hours following transfection.
(F) Phase contrast photomicrograph of E.
(G and H) Double immunodetection of transfected HA-tagged SMAK with a rabbit anti-HA antibody and of transfected Myc-RhoAN19 expressing cells using 9E10.
(I) Superposition of G and H showing colocalization of SMAK and RhoA.
(J-L) Double immunodetection of transfected HA-tagged SMAK with a rabbit anti-HA antibody and of transfected Myc-PAK expressing cells using 9E10.
(L) Superposition of J and L showing colocalization of SMAK and PAK.
Staining was performed 16 hours following transfection. Photomicrographs are shown at 400X.

Figure 13. Schematic representation of the plasmid expression vectors used in this study. The Ste20 kinase domain (black), M-NAP (grey) and ATH (white) domains are indicated. SMAK amino acid residues are indicated at the N- and C-terminal.

Figure 14. Separable SMAK Domains Induce Apoptosis and Stress Fiber Disassembly
(A and B) Immunodetection and phalloidin staining of Myc-SMAKΔC-expressing C2C12 cells 16 hours post-transfection. Extensive membrane blebbing was observed in expressing cells.
(C and D) Overexpression of the Myc-SMAKDCK63R mutant did not result in any morphological changes suggesting that it is kinase-dependent.
(E and F) Forced expression of Myc-SMAKΔN induced stress fiber dissolution in overexpressing cells as shown by phalloidin staining demonstrating that SMAK may play important roles in the regulation of actin reorganization and cell death.
(G and H) Transfection of the AT1-46 domain (pXh2973; H and I) resulted in a loss of strongly staining actin stress fibers indicating that the actin reorganizing domain of SMAK is contained within the ATH region. Photomicrographs are shown at 400X.
(I) Enhanced apoptotic response by SMAKΔC overexpression. The percentage of double positive annexin V and 9E10 cells relative to the total number of 9E10 positive cells {[(#annexinV and 9E10)/total 9E10] x 100} was evaluated at 16 and 20 hours following

transfection. The results revealed an increased rate of cell death by SMAKΔC.

(J) The indicated expression vectors were transfected into 293T cells, immunoprecipitated using 9E10 antibodies and assayed for kinase activity on myelin basic protein MBP arrow). Equivalent aliquots of protein were also subjected to Western blot analysis for normalization (upper panel). About 5- to 10-fold higher kinase activity was consistently observed with immunoprecipitated SMAKΔC.

Figure 15. Activation of SMAK through Caspase-3-Mediated Cleavage (A) Caspase-3 cleavage of in vitro translated wildtype (WT) and caspase-3 cleavage site mutant (D436N) SMAK proteins. A similar pattern of cleavage was observed in the presence of an apoptotic cell lysate from induced (2 mM b-estradiol) Rat1-Myc/ER cells. Introduction of the D436N mutation in SMAK prevented cleavage and release of the 133 and 60 kDa fragments. Addition of the caspase-3 inhibitor Z-DEVD-fmk (50 mM) to the reaction abolished cleavage.

(B) Apoptosis-induced cleavage of endogenous SMAK protein in stimulated Rat1-Myc/ER cells. The levels of SMAK fragments of 133 and 60 kDa progressively increased during the apoptotic response while the levels of the full length p220 were reduced.

(C) N-terminal-specific antibodies identified the 60 kDa fragment as the kinase domain in induced Rat1-Myc/ER cells and NIH3T3 cells exposed to apoptotic triggers. NIH3T3 fibroblasts were exposed to TNF-a (20 ng/ml) plus 10 mM pyrrolidinedithiocarbamate for 16 hours or UV-irradiated (180 J/m2) and allowed to recover in growth medium for 4 and 15 hours. Release of SMAK kinase domain was clearly evident. No reactivity to the 133 kDa fragment was observed, suggesting that it bears a C-terminal portion of SMAK.

(D) In vitro caspase-3/kinase assay on immunoprecipitated Myc-SMAK proteins. Wild type or mutant Myc-SMAK proteins were immunopurified and subjected to caspase-3-expressing lysates, or a control lysate, and assayed for kinase activity in vitro.

(E) Anti-Myc tag western blot analysis showing expression of all Myc-SMAK proteins in transfected 293 cells.

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